STUDIES ON THE WATER RELATIONS OF *COSTELYTRA ZEALANDICA*

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INTRODUCTION

Water is one of the most important components of the environment of any animal: the amount and availability of water influence the animal's behaviour, development and survival. Water relations have been studied extensively both for animals living in the wettest aquatic environments and in the driest terrestrial ones. Soil has some of the features of each of these environments, and other features of its own, but less attention has been given to the water relations of the animals living there. Soil animals, especially those which feed on cultivated plants, are particularly important because of their effect on agricultural food production. The grass grub Costelytra zealandica (White) is such an animal. In the first part of this study, C. zealandica and its soil environment, and the place of water in them, are described.

Study of the water relations of soil animals has been hindered by the lack of a simple, meaningful measure of the water in the animal-soil system. A similar problem arises in the study of water relations of plants in soil, but here progress has been made by using a thermodynamic approach. In the second part of this study, thermodynamic concepts are applied to the water relations of C. zealandica in an attempt to provide an expression of the state of water in the system in a way related to the availability of the water to the animal and the stress imposed on the animal by its shortage or excess. This theoretical framework is derived from the work of many authors. It is set out explicitly to make its possibilities, advantages and limitations clear.

The term 'water stress' refers to the physiological condition when the amount of water is unfavourable to optimum growth (Taylor, 1968). In the third part of this study it is intended to show the extent to which C. zealandica is under water stress, and to describe and demonstrate some of the adaptations and responses it uses to overcome this stress. Responses of individual animals are emphasized rather than the mechanisms of physiological responses, or population responses. The results of experiments designed to test alternative

hypotheses are analysed wherever possible by simple distribution-free statistical methods involving the fewest unwarranted assumptions. Hypotheses are rejected whenever the probability of observed results, under the hypothesis, is 0.05 or less.

Fig. 1. Costelytra zealandica third instar larva. 10x.

PART I. SETTING THE SCENE

1. The animal

1.1 General introduction

Costelytra zealandica (White), the common grass grub, is a beetle which spends all its life, apart from brief flights as an adult beetle, in the soil near the surface. Its larva (Fig. 1) is a New Zealand representative of the 'white grub' type of soil-dwelling scarabaeid larvae found in many countries. Melolontha species in Europe, Popillia japonica and Phyllophaga species in America, C. zealandica in New Zealand, and many other species, cause considerable damage to pasture by feeding on roots just below the soil surface. Such damage has occurred in exotic pasture in New Zealand for over a century (Wakefield, 1873). C. zealandica is most common in pasture but "ranges throughout the country from sea level to over 4,000 feet, in rainfall from 14 inches to over 100 inches per annum, through most soil types and associated with most plant communities except dense forest" (Given, 1966).

1.2 Systematic position

According to Given (1952) the systematic position of Costelytra zealandica is:

Order COLEOPTERA
Family Scarabaeidae
Subfamily Melolonthinae
Costelytra zealandica (White, 1846)

Hoy and Given (1952) assigned C. zealandica to the tribe Sericini, following the classification of American Melolonthinae by Hayes (1929). Given (1960) assigned it to the tribe Colpochilini, erected by Britton (1957) in his classification of Australian Melolonthinae, but Given (1966) stated that this tribal placement was "a matter of convenience and not of accuracy".

1.3 Life history

The main features of the life history of C. zealandica are well known, but detailed information on many points is still lacking. The

following account is a summary of the information given by Miller (1921), Dumbleton (1942), Kelsey (1950, 1951) and other authors.

C. zealandica completes its life cycle in one year. Eggs are laid in the soil, mainly during November and December, and only develop when in contact with moisture. First instar larvae hatch after 10-20 days and immediately begin to move about in the soil and feed on roots. They change to the second instar after about 4-5 weeks and to the third instar 5-10 weeks later. Miller (1921, 1936) reported that during winter larvae ceased feeding and hibernated deeper in the soil, but Kelsey (1950) and Given (1952) reported that larvae fed within an inch of the surface during winter, even with the soil frozen around them. By the end of September the fat content and weight of third instar larvae reach their highest levels (Perrott, Shorland and Czochanska, 1965) and the larvae cease feeding and move down in the soil to pupate. Most of them pupate in October, in earthen cells 4-10 inches below the surface. The duration of the pupal stage has been estimated as "usually not exceeding a fortnight" (Kirk, 1885) and 4-6 weeks (Kelsey, 1951).

When the beetles emerge from the pupa there are usually about equal numbers of each sex (Elliott, 1964). They remain in the pupal cell until the integument has hardened and darkened, and then burrow to the surface. For several months in summer, beetles emerge from the ground each evening "as twilight wanes" (Thomas, 1913). If there is no rain and the night is calm and warm, swarms of beetles, mainly males, then fly close to the ground for 20-30 minutes. Female beetles generally emerge after the males and since they produce a pheromone which attracts the males (Kelsey, 1967; Henzell and Lowe, 1970) they are normally mated soon after emerging and then burrow into the soil again (Fenemore and Perrott, 1970). After the flight some beetles feed on plants and trees through the night; all of them shelter during the day, usually by burrowing back into the soil. During their lifetime of several weeks, female beetles lay up to 50 eggs in several clusters three to seven inches deep in the soil. Unless the ground is bare, these are laid in the same area from which the beetle emerged (Kelsey, 1957).

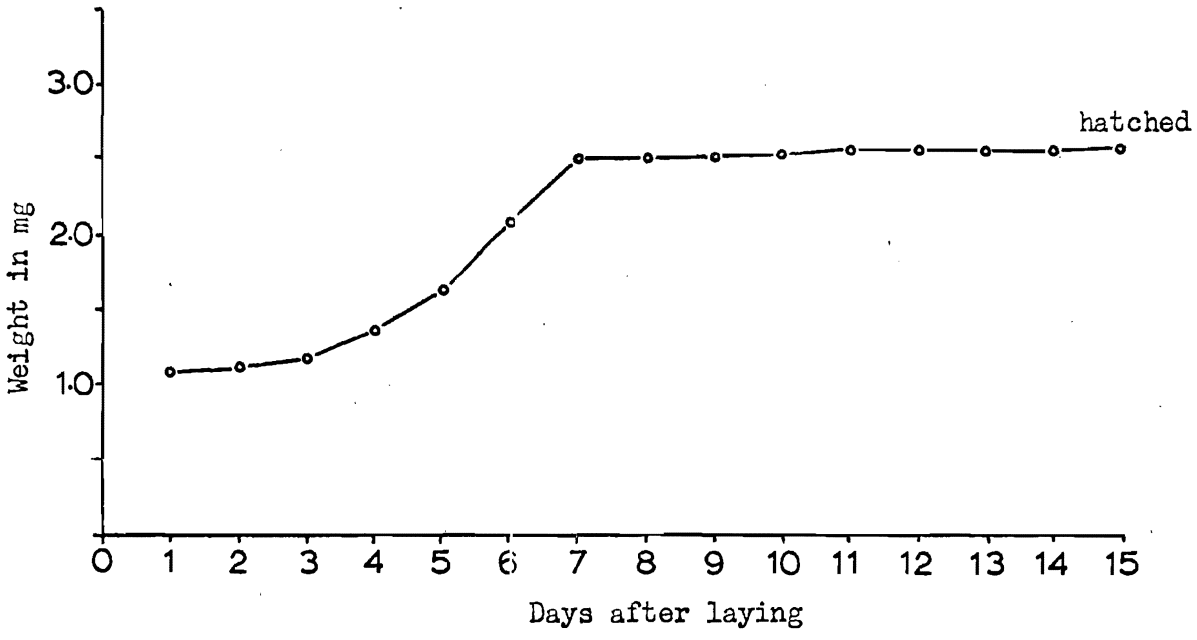


Fig. 2. Change in weight of a cluster of 21 C. zealandica eggs during development.

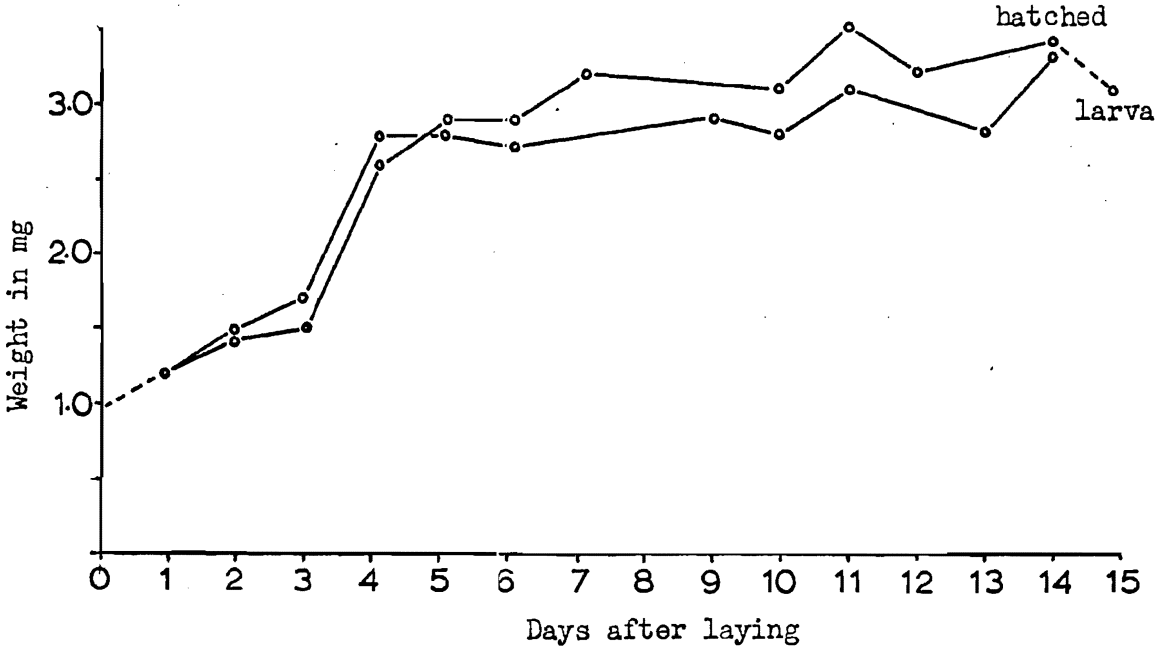


Fig. 3. Change in weight of two C. zealandica eggs during development.

1.4 Water in the animal

1.4.1 Water in the egg

To obtain newly laid eggs, female C. zealandica beetles collected after their evening flights at West Melton were kept in pots of soil, and eggs sieved out each day. The water content of these eggs (estimated from the loss in weight of a sample of 27 kept in dry air) was 50.5%, which is almost exactly the same as that found in newly laid eggs of other scarabaeid species: 49.97% in Popillia japonica (Rothstein, 1952), and 50.0% in Phyllopertha horticola (Laughlin, 1956). These authors showed that weight changes in the eggs were the result of changes in water content, and it is assumed that this is also true for C. zealandica.

Rapid absorption of water during development is a characteristic feature of scarabaeid eggs, including those of C. zealandica (Kelsey, 1950). The course of water absorption was followed by weighing eggs incubated at room temperature (18-24°C) on filter paper saturated with water. The change in the mean weight of 21 eggs in a single cluster is shown in fig. 2, and the change in weight of two other eggs weighed individually are shown in fig. 3. All these eggs developed and hatched normally. The two weighed individually increased to 275% and 292% of their initial weights, but the cluster increased to only 235% of its initial weight, possibly because few of the eggs in it were in direct contact with the water in the filter paper.

The characteristic pattern of water absorption: slow initial gain followed by a short period of rapid absorption after which little change takes place until hatching, has been recorded from eggs of many scarabaeid species and studied in more detail in Melolontha melolontha (Hurpin, 1956), Aphodius howitti (Maelzer, 1961), and the two species already mentioned. Although these species live in a range of climates their absorption of water does not vary and therefore must depend more on common properties of the eggs rather than the environment. Laughlin (1957) and Lower (1957) discussed internal changes in scarabaeid eggs and possible mechanisms regulating water absorption. Surface properties and permeability of the outer layers of C. zealandica eggs, which also influence the rate at which they absorb

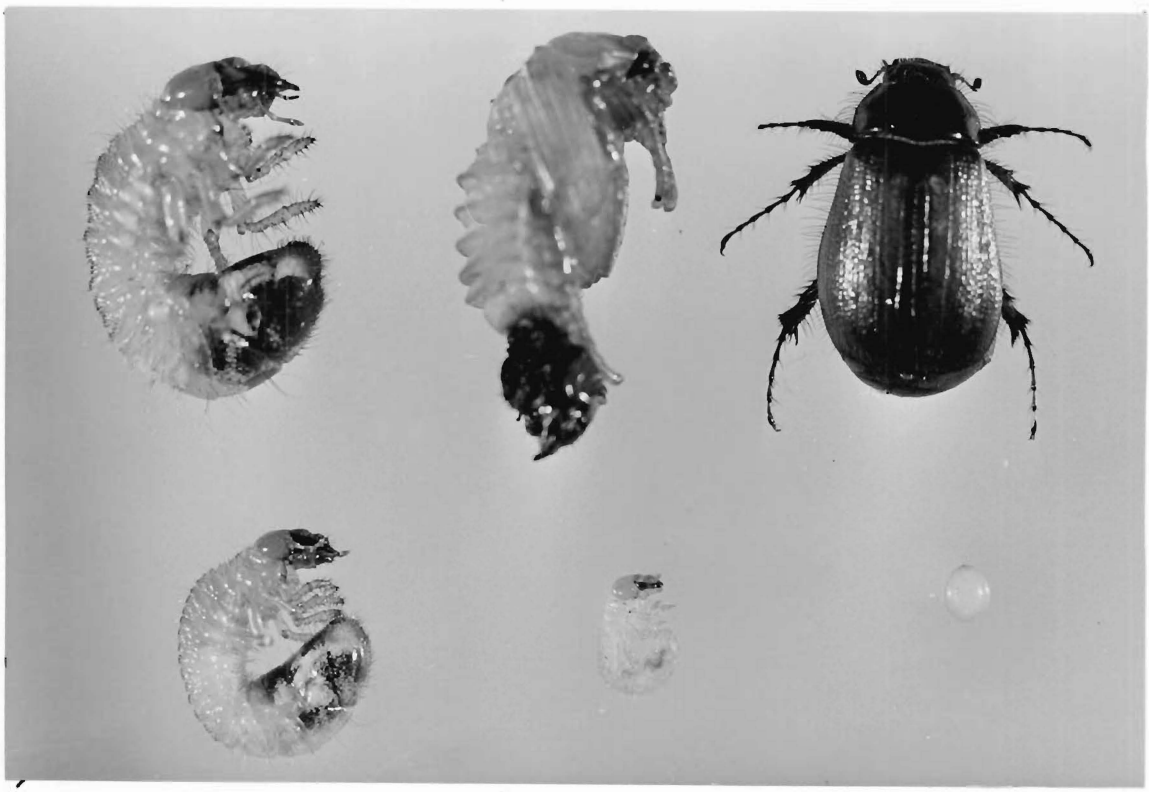


Fig. 4. The stages of the life cycle of Costelytra zealandica. 5x.

Third instar larva pupa adult beetle

Second instar larva first instar larva egg

water, are discussed in sections 1.6.6 and 1.6.7.

1.4.2 Water in the other stages

Unlike the egg, the larval, pupal and adult stages have fairly constant water contents. Measurements of the mean water contents of samples of larvae of C. zealandica (determined by drying them for 24 hours at 105°C), and of other scarabaeid species (from the literature) for comparison are listed in Table 1.

Table 1. Water contents of C. zealandica and other scarabaeid larvae.

Species	Instar	Water content	Author
C. zealandica	2	82.7%	
	3	75.5%	
Phyllopertha horticola	2	88.0%	Laughlin (1956)
	3	79.1-84.2%	
Serica brunnea	3	73.2-83.5%	Fidler (1936b)
Aphodius howitti	3	77%	Maelzer (1961)
Popillia japonica	3	77.6%	Ludwig (1946)
Phyllophaga sp.	3	77.7%	Sweetman (1931)
Melolontha hippocastani	3?	79.2%	Sacharov (1930)

As before, all the species are remarkably similar - again suggesting that they have common properties which promote control of water content by the animal rather than the environment.

The integument of the egg is quite uniform and water apparently diffuses through the whole surface area, but in the other stages different parts of the integument have specialised functions and properties which influence the movement of water through them. This applies particularly to the spiracles, which are therefore considered separately from the rest of the integument. Other modified regions of the integument such as the intestine are not dealt with in this study.

1.5 General morphology

The external appearance of all the stages of C. zealandica is illustrated in figs 1 and 4. The third instar larva was described by

Given (1952), its intestine by Allison (1969) and the adult beetle by Hoy and Given (1952). The only other published information on morphology of C. zealandica is in notes on sexual dimorphism in the larval instars (Elliott, 1964), the pupa (Brown, 1967) and the adult beetle (Kelsey, 1965).

Many Scarabaeidae, mainly from the subfamilies Melolonthinae and the closely related Rutelinae, have been studied because of their importance as pests. The most comprehensive morphological study is that of Lupo (1946) on the morphology, anatomy and histology of all stages of Anomala ausonia (Rutelinae). Detailed morphological studies of species of Melolonthinae have been made by Fidler (1936a), Subklew (1938) and Butt (1944), who dealt with external structure, and Grandi (1925) and Jepsen (1937) who also dealt with some aspects of internal anatomy. C. zealandica closely resembles the species described by these authors. Some aspects of the morphology and physical properties of its integument will be described in detail because of the importance of the integument as a route of water gain or loss.

1.6 Morphology and physical properties of the integument

The integument separates the animal from its surroundings. In arthropods it is formed from a single epithelial layer which deposits on its outer surface a durable acellular cuticle with low permeability to water (Richards, 1951). A thin basement membrane separates the epithelial layer from the body cavity. The cuticle is divided into an outer epicuticle and inner procuticle comprising three layers: exocuticle, mesocuticle and endocuticle (Richards, 1951; Schatz, 1952). The wall of the egg has a different structure: an outer chorion, which is laid down in the ovary, and inner blastoderm layers, which are modified during development of the egg (Johannsen and Butt, 1941).

Two techniques were used to study the microscopic structure of the integument of each stage of C. zealandica. The light microscope was used to examine the histology of sections 7 µm thick cut from animals fixed in Sanfelice's fluid, embedded in paraffin wax and stained by the technique of Lower (1955). A Cambridge 'Stereoscan' Scanning Electron Microscope (hereafter referred to as the SEM) was

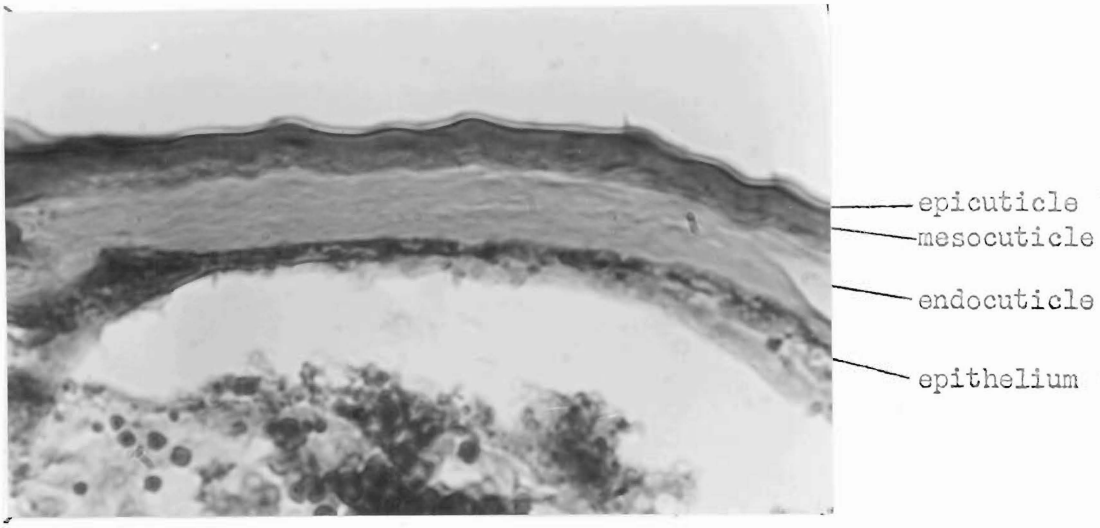


Fig. 5. Section of cuticle on the abdomen of a third instar larva. 1,000x.

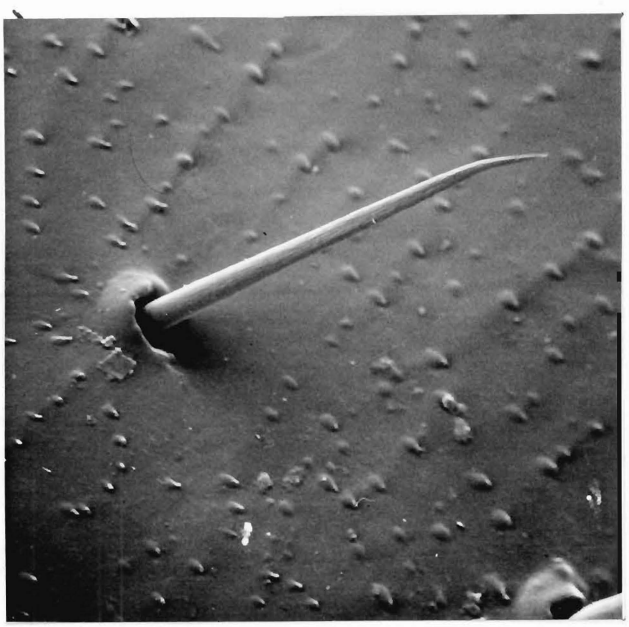


Fig. 6. SEM photograph of the cuticle surface and a spine on the abdomen of a third instar larva. 1,000x.

used to examine the surface structure of whole animals. These animals were prepared for SEM examination by dropping them into Freon 12 or directly into liquid nitrogen, freeze drying at -70°C, and coating under vacuum with layers of carbon, then gold-palladium, each approximately 20 nm thick. These layers conduct away the electric charge which builds up on the specimen (Echlin, 1968).

1.6.1 Morphology of the larval cuticle

Except for the hard sclerotised head capsule, mouthparts, spiracular plates and other such parts, the cuticle of the larva is thin, soft and folded. The internal organs are visible through it, especially in the last abdominal segments. Setae of various sizes are scattered over most of the cuticle. On the dorsal part of the first six abdominal segments many short setae are arranged in distinct areas.

A section of cuticle from the abdomen of a third instar C. zealandica larva is shown in fig. 5. There are three distinct cuticular layers outside the epithelium which correspond to those Lower (1957) described in the cuticle of the scarabaeid Aphodius howitti. The outer unstained layer, the epicuticle, is less than 1 μm thick and is missing from parts of the cuticle. The two other layers are well differentiated by the staining technique. The outer of the two, the mesocuticle, is about 5 μm thick and stains red, while the inner layer, the endocuticle, stains blue-green. The endocuticle has a laminar structure and varies in thickness, but is usually about 10 μm thick. The cuticles of C. zealandica and A. howitti are quite similar in structure but different in thickness. Comparing the above measurements with those of Lower (1957), the epicuticle and endocuticle of C. zealandica are each only half as thick as those of A. howitti, while the mesocuticle is 5 μm thick in both species.

Fig. 6 is an SEM photograph of the cuticle surface in the same area of the abdomen as shown in the section in Fig. 5, and at the same magnification. The surface is particularly smooth and featureless apart from the rows of minute tubercules. These are found on most of the soft cuticle of the three larval instars except on parts of the

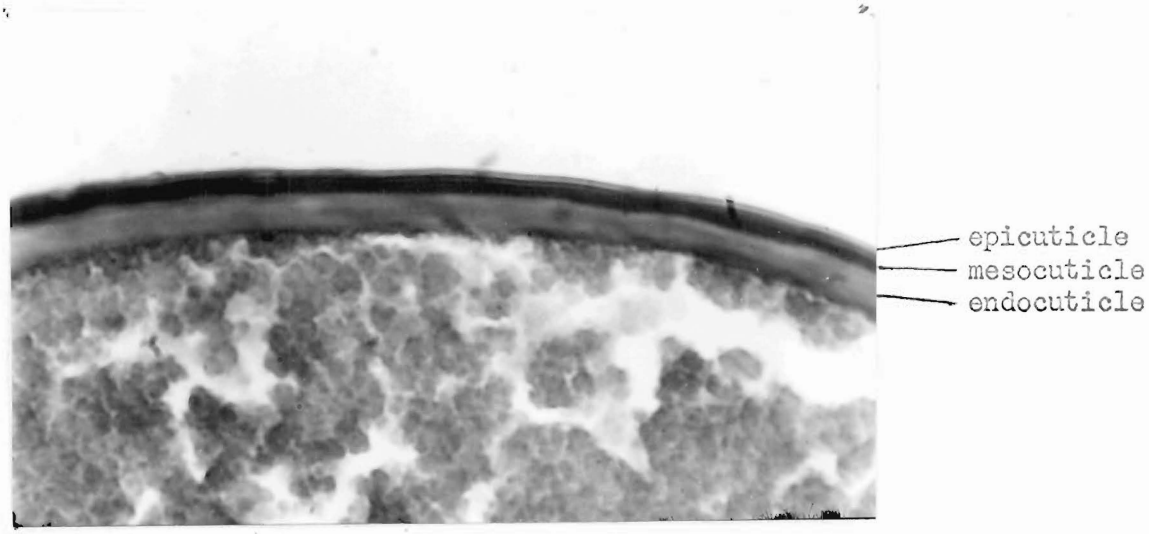


Fig. 7. Section of cuticle on the abdomen of a pupa. 1,000x.

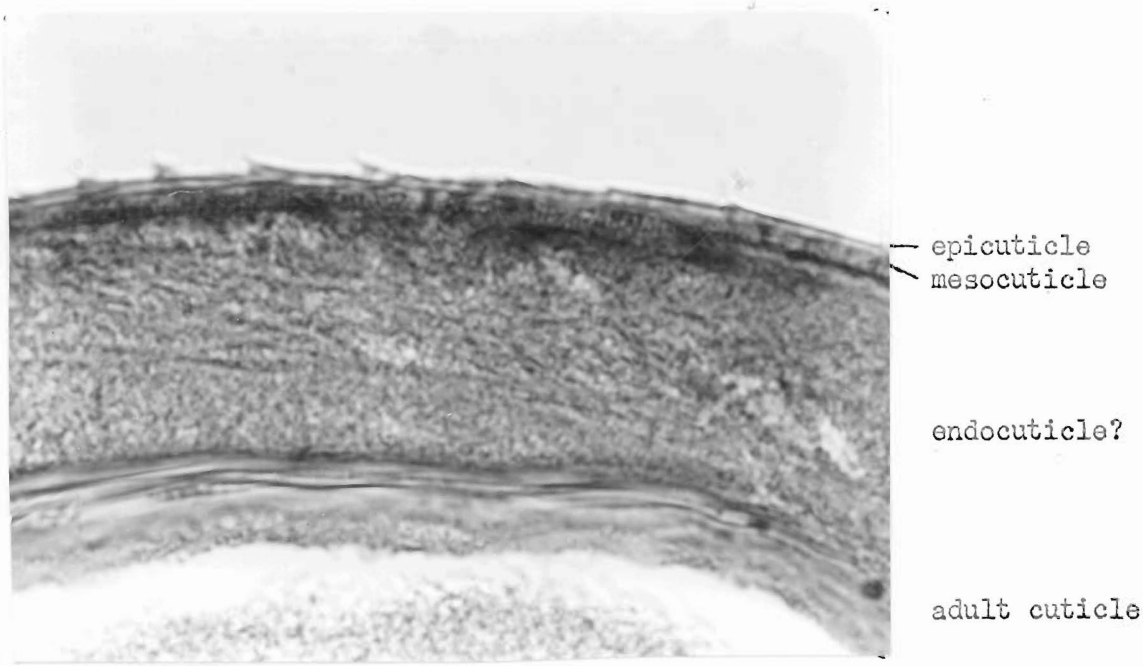
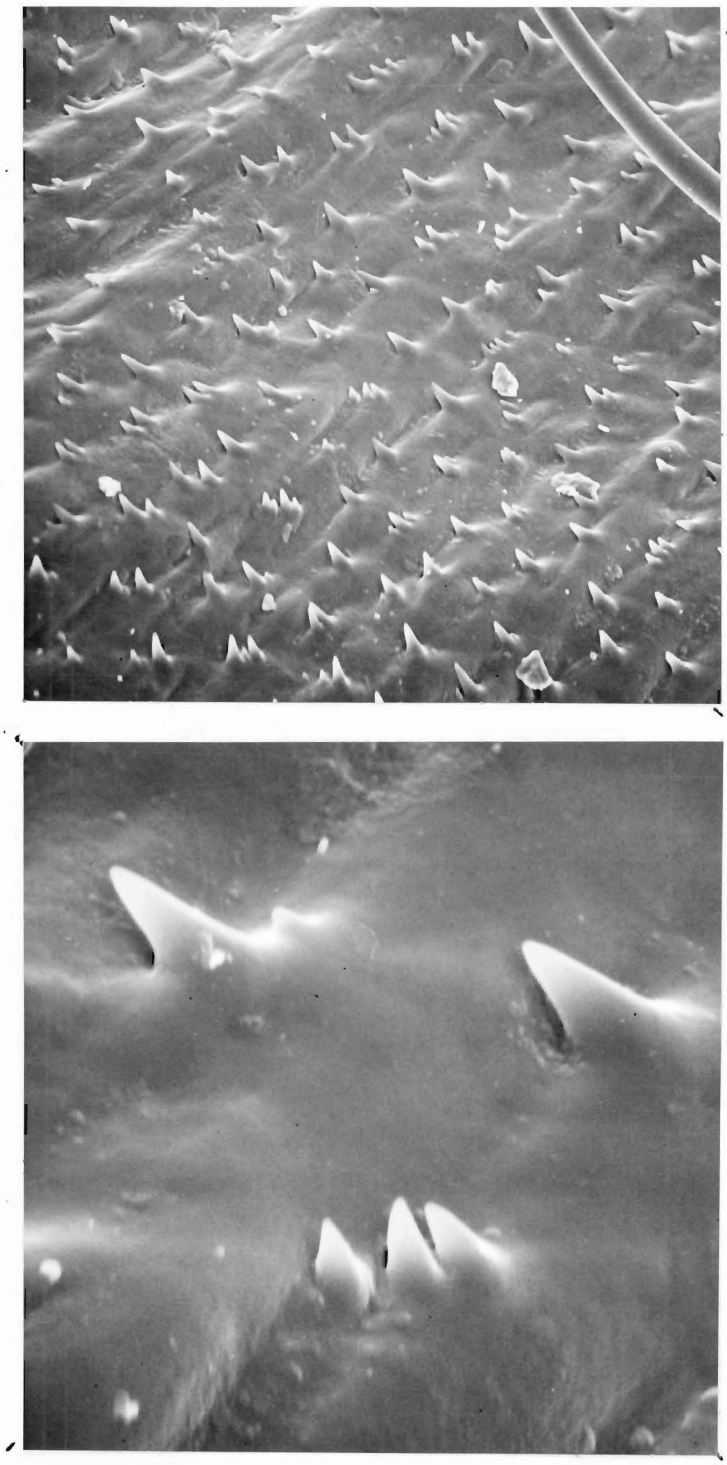


Fig. 8. Section of cuticle near a spiracle on the abdomen of a pupa, showing the developing adult cuticle. 1,000x.



Figs 9 (upper; 1,000x) and 10 (lower; 5,000x). SEM photographs of the cuticle on the abdomen of a pupa.

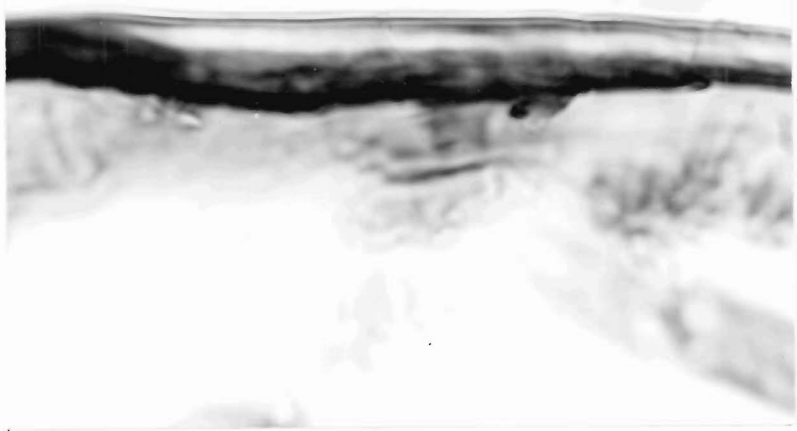


Fig. 11. Section of cuticle on the abdomen of an adult beetle. 1,000x.

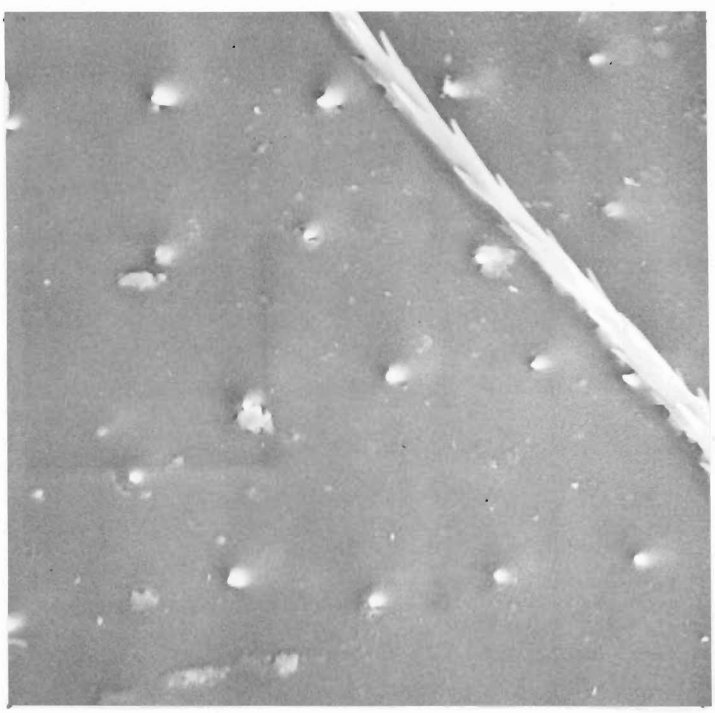


Fig. 12. SEM photograph of cuticle surface and part of a long spine on the abdomen of an adult beetle. 2,100x.

last abdominal segments which are quite smooth. They correspond to the verruculae found by Lower (1957) on the epicuticle of A. howitti.

1.6.2 Morphology of the pupal cuticle

The pupa is soft and its cuticle, unlike that of the larva, is easily damaged by handling. The external lips of some of the spiracles are the only sclerotised areas, and there are no setae.

Sections of the pupal cuticle (figs 7 and 8) show that the basic structure is similar to that of the larval cuticle. The epicuticle is a clear brownish unstained layer less than 1 μm thick which in some areas bears minute spines (fig. 8) like the smallest of the microtrichia Lower (1957) found on A. howitti. These are seen more clearly in SEM photographs of the cuticle surface (figs 9 and 10). The red-stained mesocuticle varies from 2-5 μm thick. In most of the cuticle the blue-stained endocuticle is a distinct layer about 5 μm thick. Around the spiracles, where the developing adult cuticle lies close beneath the pupal cuticle (fig. 8) there is a layer 30 μm thick between the pupal and adult mesocuticles which stains purple, grading into blue just below the pupal mesocuticle. How much of this layer is endocuticle or derived from endocuticle is not clear. Apart from these areas, the pupal cuticle has a total thickness of about 8 μm - half the thickness of the larval cuticle.

1.6.3 Morphology of the adult cuticle

The cuticle of the adult is sclerotised and hard, especially on the head, thorax and legs. The only beetles available for sectioning had been stored in alcohol which hardens the tissues even more, so section cutting was not very successful as most of the sclerotised layer broke away from the rest of the cuticle. Attempts at softening the cuticle in 3% phenol solution or in warm cupric trinitrophenol (Petrunkevitch, 1933) gave no better results.

The cuticle appeared to be softer near the fold between abdominal segments and sections of this region were less fragmented than other parts. Such a section is shown in fig. 11. The structure of the abdominal cuticle of the adult C. zealandica is similar to that

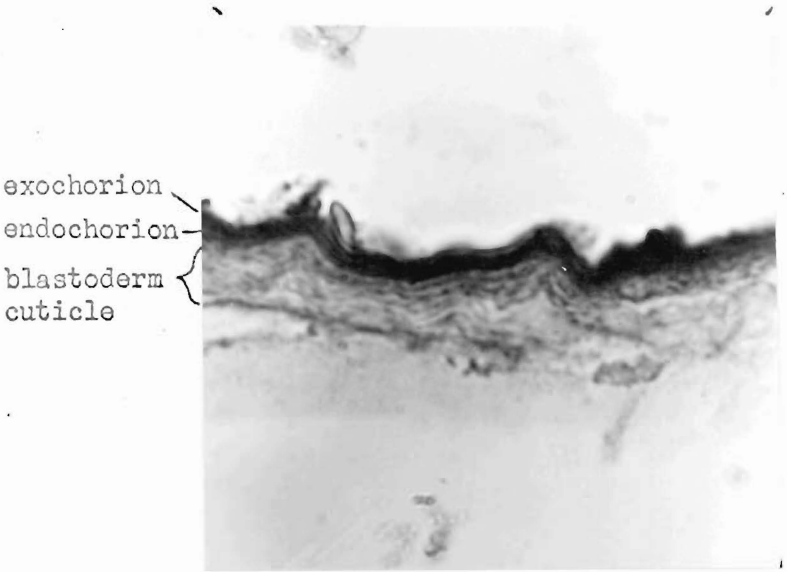


Fig. 13. Section of the outer membranes of an unswollen egg. 1,000x.

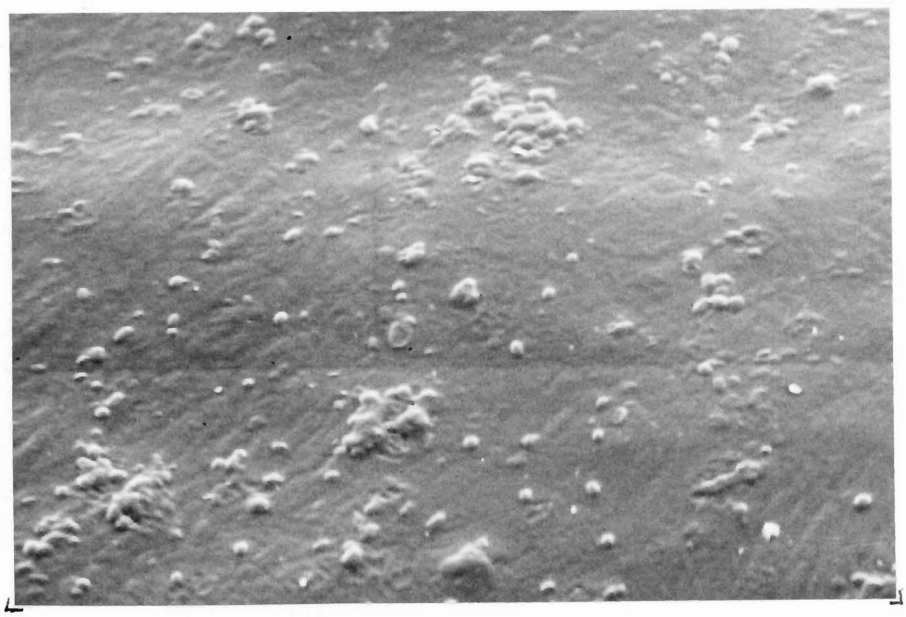
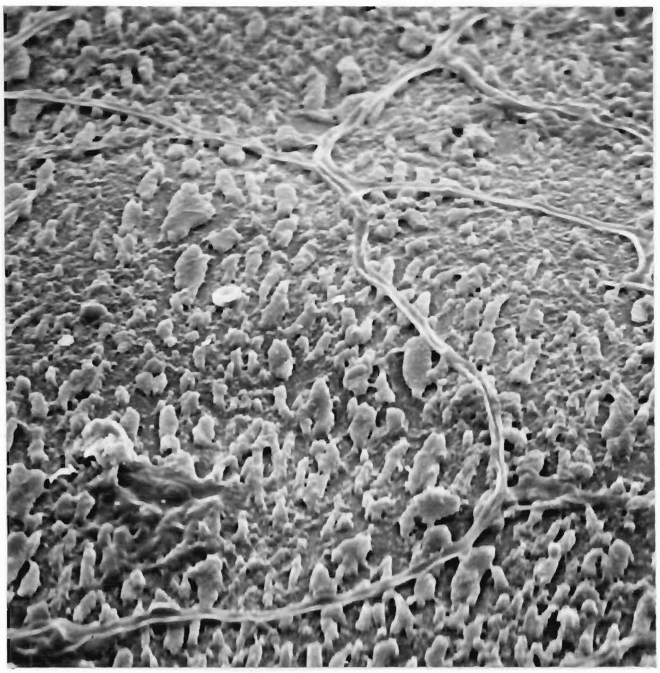
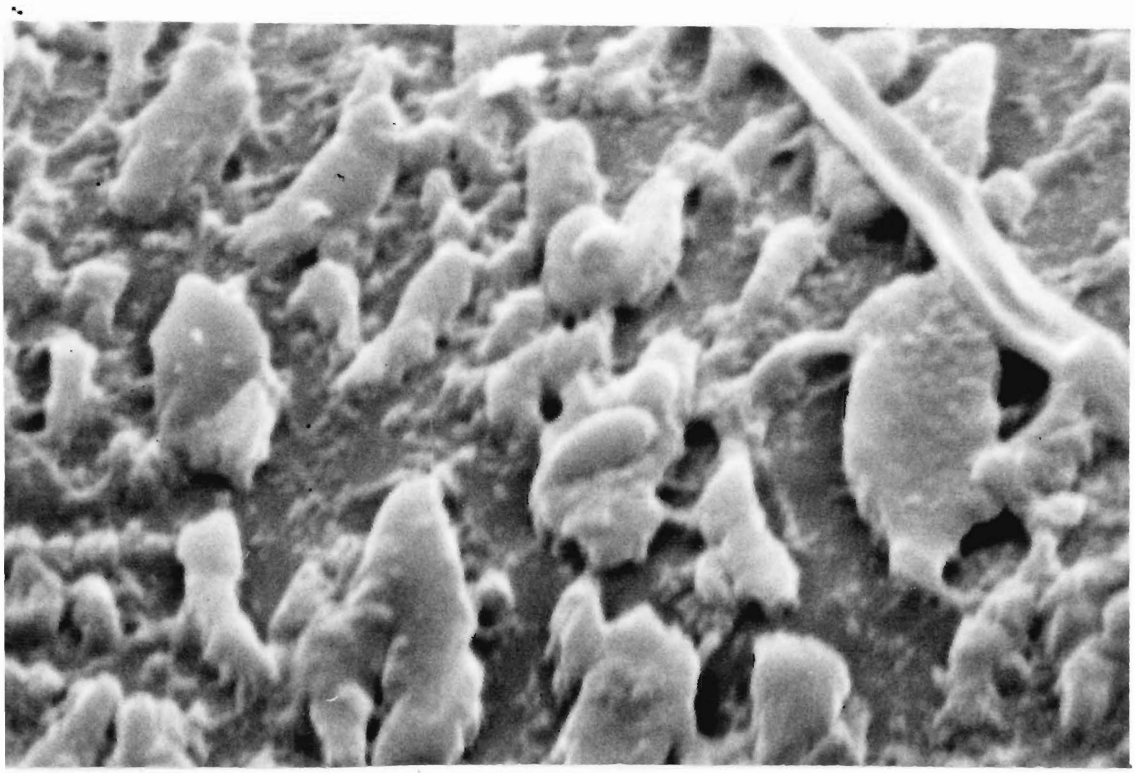


Fig. 14. SEM photograph of the surface of the chorion of an unswollen egg. 2,000x.





Figs 15 (upper; 2,000x) and 16 (lower; 10,000x). SEM photographs of the surface of the chorion of a swollen egg.

of the other stages apart from the sclerotised exocuticle. The outer layer, the epicuticle, is less than 1 µm thick. Under this is the exocuticle, also clear and unstained, but with the characteristic brown colour of sclerotised cuticle. This layer is about 5 µm thick and grades into the mesocuticle, stained red in the sections, which is also about 5 µm thick. Near the intersegmental fold the mesocuticle becomes thicker at the expense of the exocuticle. The blue stained endocuticle varies in thickness but is generally about 10 µm thick.

In some sections there were minute flattened spines on the epicuticle. The SEM photograph of the surface of the cuticle of the abdomen (fig. 12) shows these spines in regular rows (and also part of a larger spine or hair which has smaller spines along its length). Lupo (1946) found similar spines on the cuticle of the scarabaeid Anomala ausonia and described the histology of the cuticle, but gave too few details to show any more than a general similarity with C. zealandica.

1.6.4 Morphology of the egg coverings

The outer membrane of the egg of C. zealandica is stretched taut by the internal pressure of the egg. It is translucent and the brown sclerotised tips of the mandibles become visible through it as the embryo approaches full development. Sections (fig. 13) showed two main layers in the outer membrane of unswollen eggs. The outer chorion, is made up of two layers 0.75 µm and 2 µm thick, stained different shades of red - the exochorion and endochorion. Underneath is an undifferentiated blue stained layer mainly about 5 µm thick. Lower (1957) called this layer the blastoderm cuticle.

The SEM shows that the surface of the chorion of the unswollen egg is smooth with an irregular wavy pattern (fig. 14) (the straight furrows seen across some of the SEM photographs including fig. 14 are artifacts produced by the electron beam of the SEM). The chorion of the swollen egg appears quite different. Figs 15 and 16 show that it is covered with irregular nodules rising from a smooth surface. The threadlike structures lying across the chorion appear to be fungal

hyphae, like those found by Gray (1967) during SEM examination of soil, which have collapsed during the freeze drying process.

There are several types of egg surface structure in the Scarabaeidae. The chorion of the Aphodius howitti egg remains smooth and unchanged throughout embryogenesis (Lower, 1957), but in other species, including Melolontha melolontha (Hurpin, 1956) and M. vulgaris (Vogel, 1950), the exochorion splits up into separate pieces as the egg swells while the elastic endochorion stretches. The SEM photographs indicate that this also occurs in C. zealandica. However the exochorion of Phyllopertha horticola is 'granulated' even on newly laid eggs and remains the same throughout development (Laughlin, 1957). Similarly, on many Anomala species the chorion is always covered with granules or tubercles (Lupo, 1946; Nakashima, 1948, 1952). The species with each of these types of surface structure on their eggs belong to a different subfamily of Scarabaeidae: Aphodiinae, Melolonthinae and Rutelinae respectively.

No special structures for water absorption were seen in C. zealandica eggs, nor have any been found in other Scarabaeidae; water is absorbed through the entire surface.

1.6.5 Water movement and physical properties of the integument

The movement of water between the animal and its environment may be divided into two steps: through the integument, and away from the integument through the boundary layer around it. The resistance to the movement of water through the integument itself depends on the permeability of the integument. The resistance to water movement away from the surface of the integument is much greater in the vapour phase than in the liquid phase and may therefore be increased by surface properties of the integument which reduce wetting (contact with liquid water). The property of a surface which is used to define its wettability is the contact angle between the liquid-vapour interface and the solid surface (Zisman, 1964). The overall rate of movement may be limited by either step, and thus is said to be either membrane-limited or vapour-limited (Beament, 1961).

1.6.6 Permeability, transpiration and abrasion of the cuticle

Water tends to move through a membrane when the activity of the

Table 2. Weight loss and transpiration rate of C. zealandica during 24 hours at 75% relative humidity.

Stage	Sample size	Mean initial weight (mg)	Mean % loss	Standard deviation	Transpiration rate
egg	9	0.95	40	7.1	40
larva					
(3rd instar)	19	110.1	39	9.5	125
(prepupal)	19	139.7	20	3.7	80
pupa	13	157.9	40	8.2	175
adult					
(male ex lab)	20	65.5	26	7.8	55
(male ex field)	10	59.4	35	7.8	65
(female ex field)	29	54.9	32	8.8	55

Standard deviation of egg sample estimated from means of subsamples.

Transpiration rates expressed in $\mu g\ cm^{-2}\ hour^{-1}\ (mm\ Hg)^{-1}$.

water on each side is different, as indicated by a difference in hydrostatic or osmotic pressure, concentration, relative humidity etc. The rate of movement depends on the permeability of the membrane. This concept is widely used but there is no standard definition or unit (Stein, 1967). Measuring the activity at each side of the membrane is difficult, particularly when there is a gradient of activity away from its surface (i.e., a boundary layer). In most investigations of the permeability of insect integuments, the water activity has been measured or controlled at a distance from the integument. Such experiments in fact measure the overall rate of evaporation of water from the insect (the transpiration rate) under the particular activity difference, rather than the rate of movement through the integument alone. If evaporation is vapour-limited, for instance when the integument is highly permeable or when the air around it is insufficiently stirred, these rates may be quite different. However the overall rate of gain or loss of water is more significant in the life of the animal than the actual permeability of the integument, so the transpiration rate is a more useful measure here.

The rate of transpiration from each stage of C. zealandica in conditions much drier than it would normally encounter was determined by measuring the rate of loss of weight of animals kept at constant 75% humidity. A few active third instar larvae lost some weight by defecation but this was negligible and weight loss is equated with water loss in all stages for this experiment. A simple constant humidity apparatus was made from a closed container with saturated sodium chloride solution to maintain the relative humidity at 75% (Winston and Bates, 1960) and an electric fan to stir the air. The whole apparatus was kept in a room maintained at 24°C. The animals were placed in separate compartments of ice-cube trays with wire gauze floors and each weighed before and after 24 hours in the apparatus.

The mean and standard deviation of the percentage weight loss of each sample, and estimates of the corresponding transpiration rates are given in Table 2. The surface areas used for the transpiration

Fig. 17. SEM photograph of maxillary palp of third instar larva showing scratches in sclerotised cuticle. 1,150x.

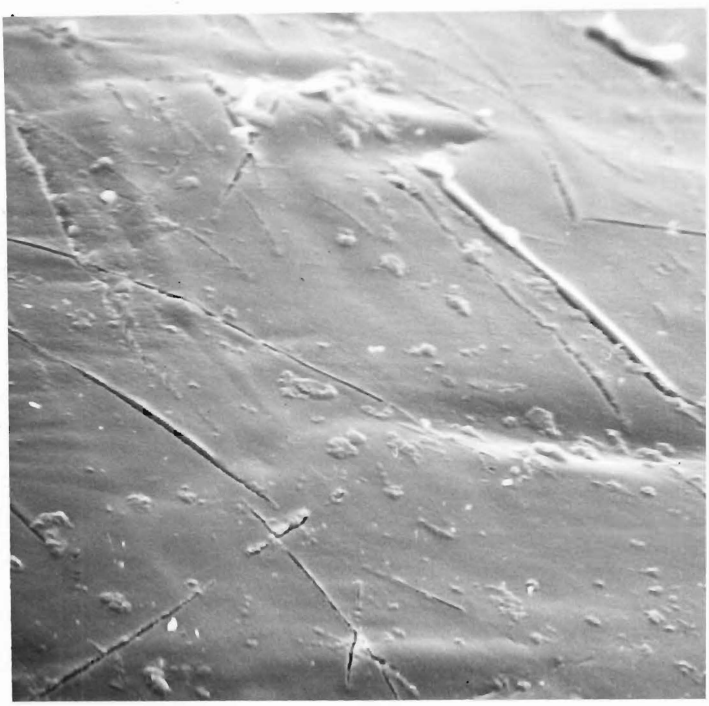


Fig. 18. SEM photograph of surface of head capsule of third instar larva showing scratches in sclerotised cuticle. 1,800x.

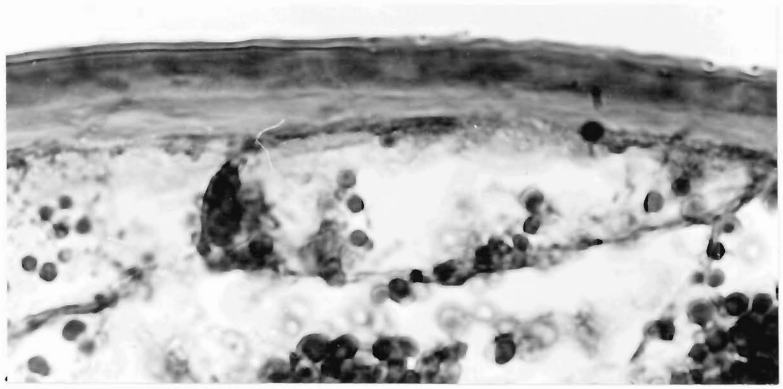


Fig. 19. Section of cuticle of third instar larva showing damaged epicuticle on right. 1,000x.

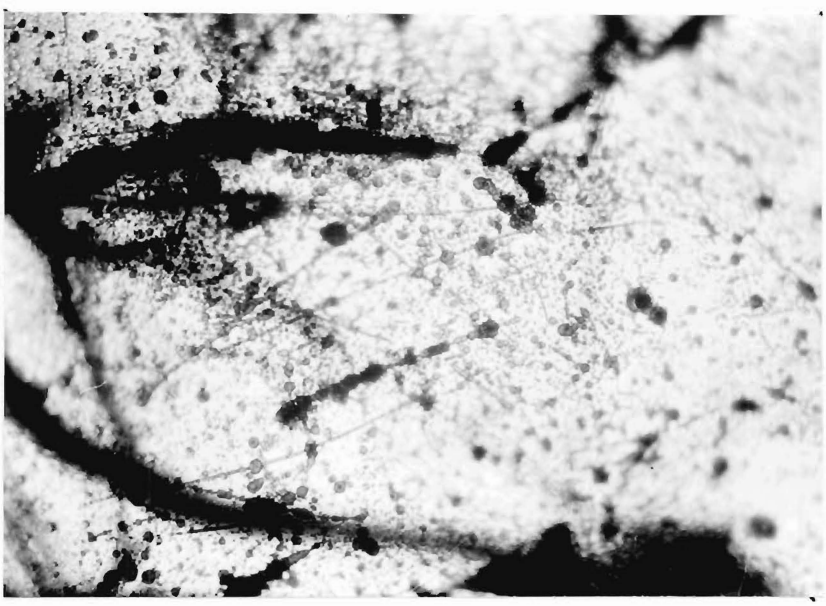


Fig. 20. Surface of abdominal cuticle of third instar larva with natural abrasion displayed by treatment with ammoniacal silver nitrate. The fine spots and lines of precipitated silver show where the epicuticle is damaged. 150x.

rate estimates are based on considering the egg as a sphere 1.5 mm in diameter, and the other stages as cylinders with these dimensions: larva 15 mm long, 4 mm diameter; pupa 11 mm long, 5 mm diameter; adult 9 mm long, 5 mm diameter. The other variables were not controlled closely enough to warrant using more exact surface areas. Transpiration rates are expressed in terms of the saturation deficit (6 mm Hg at 75% relative humidity at 24°C) to simplify comparison with published estimates, such as those given by Bursell (1964).

The transpiration rates determined for each of the stages of C. zealandica are high compared with those of other insects and are within the range where evaporation becomes vapour-limited (Beament, 1961). The measured rates can not be taken as representative of those of animals in soil, where air movement and other conditions may be different from the experimental conditions, but transpiration will remain vapour-limited unless the animal is in contact with liquid water. Such high transpiration rates are typical of soil insects, because of "abrasion of the cuticle by soil particles" (Wigglesworth, 1945). Abrasion has a significant effect on the permeability of insect cuticle because most of the resistance to diffusion of water through it is in its outermost layer - the epicuticle (Beament, 1961). Abrasion also allows disease fungi to penetrate the cuticle (Vago, 1959).

SEM photographs of C. zealandica larvae show some signs of abrasion of the cuticle. The soft cuticle appears smooth and undamaged (fig. 6) but there are obvious scratches on the more rigid sclerotised parts such as the maxillary palp (fig. 17) and the head capsule (fig. 18). Damage to the soft cuticle does occur, as sections often have parts of the epicuticle missing (fig. 19) and a clear pattern of scratches is revealed on the cuticle of the abdomen by the argentaffin test (fig. 20), in which polyphenols in the deeper epicuticular layers reduce ammoniacal silver nitrate to metallic silver where the surface layer has been abraded away (Richards, 1951).

1.6.7 Contact angles and wettability of the integument

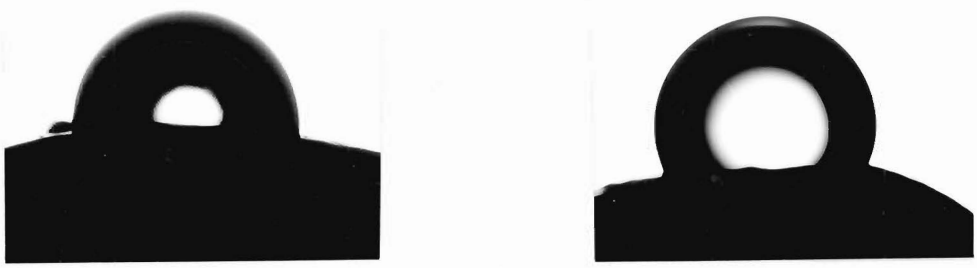
In wetter soils, C. zealandica may be in contact with liquid water. The extent to which water spreads over its cuticle depends on the contact angle between water and cuticle. The lower the contact angle, the more the water spreads. If the angle is high enough the water remains in "droplets which run over the surface fairly easily and are not difficult to shake off" (Adam, 1958). Normally the contact angle is larger when the liquid is advancing across a surface (advancing contact angle) than when it is receding (receding contact angle). It is also affected by the roughness of the surface - in general, roughening a surface increases contact angles above 90° and decreases those below 90° (Zisman, 1964; Gray, 1965).

Wetting of insect cuticle by liquids has been mainly studied in connection with contact insecticides and the way spray droplets spread on cuticle (Wilcoxon and Hartzell, 1931; Pal, 1951). Holdgate (1955) investigated surface properties as an indication of cuticle structure and measured contact angles under carefully controlled conditions using uncontaminated water on clean cuticle. Both cuticle and water on an animal in soil are normally contaminated, thus to find how water behaves on the cuticle under normal conditions, the relevant contact angles are those measured on contaminated insects, and Holdgate's (1955) precautions are not needed. Thus animals were taken from soil and were not subjected to any cleaning procedure apart from rolling on clean filter paper to remove the larger soil particles. The SEM photographs show that animals cleaned in this way are still quite heavily contaminated, but this is their normal condition.

Contact angles of water on various areas of the cuticles of the stages of C. zealandica were found from photographs of profiles of drops of water in air and bubbles of air in water resting on the cuticle. Small drops (less than 1 mm diameter) were used since these give the advancing contact angle (Mack, 1936). By the same argument small air bubbles in water should give the receding contact angle. The contact angle was calculated from the dimensions of the drop or bubble when the cuticle was flat or regularly curved (Mack, 1936;

Egg

Third instar larva: abdomen



Third instar larva: head capsule

Fig. 21. Contact angles of water on C. zealandica cuticle.
Left: drops showing advancing contact angle.
Right: bubbles showing receding contact angle.
(Actual size of all drops and bubbles less than 1 mm.)

Pupa

Adult beetle: abdomen

Adult beetle: elytron

Fig. 22. Contact angles of water on C. zealandica cuticle.
Left: drops showing advancing contact angle.
Right: bubbles showing receding contact angle.
(Actual size of all drops and bubbles less than 1 mm.)

Mack and Lee, 1936), otherwise the angles were measured from the photographs. The flattest areas were chosen and hairs and spines avoided where possible. The insect was tilted until the drop and the surface it rested on could be seen in profile before the photograph was taken. This was difficult and not many of the photographs showed clear profiles suitable for determining the contact angles. Some of these are shown in figs 21 and 22. Means and ranges of the contact angles determined are listed in Table 3.

Table 3. Contact angles of water on the cuticle of C. zealandica.

Stage	Area of cuticle	Advancing angle (degrees)		Receding angle (degrees)	
		Mean	Range	Mean	Range
egg		107	94-116	98.5	86-114
larva (third instar)	abdomen	86.5	80-89	53	50-59
	head capsule	102	95-109	89.5	82-93
pupa	various areas on thorax and head	91	84-99	72	65-77
adult	abdomen	95	92-98	76	73-80
	elytron	88	82-98	58	53-61

The contact angles measured on C. zealandica are generally lower than those measured on terrestrial insects by Holdgate (1955), which is probably the result of contamination. Except on the egg and the sclerotised larval head capsule, the advancing angles are close to 90°, and the receding angles rather lower, which indicates that water on the cuticle of C. zealandica will tend to remain in separate drops rather than spread, although such drops will not shake off easily. Hence the cuticle of the animal in its space in the soil will generally remain dry. Maintenance of a layer of air around the animal in this way is important because it ensures that water movement through the cuticle, made comparatively permeable by abrasion, remains vapour-limited.

Surface properties have special significance for both the egg and

the sclerotised larval cuticle, which have discrepant contact angles. The effectiveness of the larval spiracles depends on the contact angles on their sclerotised cuticle for reasons which will become clear when the structure of these spiracles has been described. The high contact angles on the egg could be a disadvantage since dry egg surfaces could restrict absorption of water during their development.

Although the eggs used for measurement of contact angles had been kept on damp filter paper, their surfaces were relatively dry. Newly laid C. zealandica eggs are covered with a "clear sticky fluid that makes them adhere closely to dry surfaces" (Kelsey, 1951). Many authors mention such a secretion binding the soil about the eggs of Scarabaeidae. In his account of the biology of the Rutelinid Anomala ausonia, Lupo (1949) described this secretion as being emitted by the colleterial glands of the female beetle. He deduced that by causing soil particles to adhere to the chorion it ensured that the water necessary for the egg's development could be taken up from the surrounding soil. Other functions have also been proposed for the secretion of the colleterial glands of Melolonthinae: Nonveiller (1958, 1960) suggested that in Miltotrogus aequinoctialis and other species it contained a sex attractant.

As well as attaching the eggs closely to the surrounding soil the secretion on the eggs may affect their wettability. Naturally occurring macromolecular materials such as water soluble proteins and gums are often surface active agents (Moilliet, Collie and Black, 1961), substances which are adsorbed at interfaces and decrease surface tensions and contact angles. Unfortunately no direct test was made but some observations suggest that the secretion on C. zealandica eggs has surface active properties. The eggs kept on damp filter paper floated on water unless they were pushed below the surface. A particle which floats although it is denser than water is supported by a force proportional to the sine of the contact angle of water on the particle and the surface tension of the water (Gaudin, 1957). Thus lowering the contact angle or the surface tension by adding a surface active agent helps the particle to sink. According to Kelsey (1951) "When freshly laid, eggs sink in water ...

Fig. 23. SEM photograph of third abdominal spiracle of third instar larva showing crescent shaped spiracular plate and central bulla. 250x.

if the egg surfaces dry out and the eggs are placed in water they will float for some time before sinking". A surface active agent in the secretion on the freshly laid eggs would explain these observations. Such an agent would promote contact with liquid water and thus aid absorption of the water necessary for development, despite the contact angle being higher on the egg than on any other stage.

1.7 Morphology and physical properties of the spiracles

Since much of the water lost by insects evaporates from the tracheal system, the spiracles at the openings of the tracheae can have an important influence on the rate of water loss (Mellanby, 1934). The larval, pupal and adult stages of C. zealandica all have spiracles with quite different structures.

1.7.1 Morphology of the larval spiracles

Third instar C. zealandica larvae have pairs of spiracles on the prothoracic segment and the first eight abdominal segments (Hoy and Given, 1952). They are all less than 0.2 mm across and so are visible to the naked eye only as brown spots (fig. 1).

The external structure of the third abdominal spiracle of a third instar larva as seen under the SEM is shown in fig. 23. The crescent shaped spiracular plate surrounding the central bulla is pierced by about 200 fine slit-like aeropyles. Between the spiracular plate and the bulla is the external scar of the ecdysial tube, through which the previous instar's tracheal lining was withdrawn at ecdysis. Spiracles with these features are characteristic of scarabaeid larvae and have been described in detail by Lotz (1962) and Hinton (1967). The other spiracles of third instar C. zealandica larvae are similar to the one in fig. 23 except for slight variations in size and shape. The length of the spiracular plate decreases regularly from about 200 μm in the thoracic spiracle to about 90 μm in the eighth abdominal spiracle, except for the seventh abdominal spiracle, which at 140 μm across is rather larger than its neighbours. The number of aeropyles varies with the size of the spiracle. The open side of the crescent of the spiracular plate faces posteriorly on the thoracic spiracle and anteriorly on all the

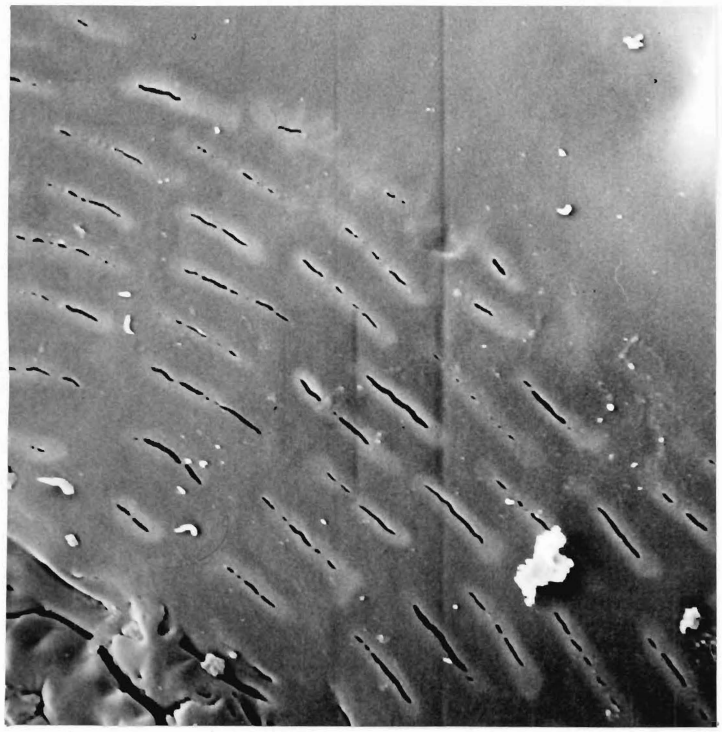


Fig. 24. Enlargement of part of the spiracular plate of the spiracle in Fig. 23., showing the aeropyles. 2,400x.

Fig. 25. Enlargement of Fig. 24. showing aeropyles. 20,000x.

Fig. 26. Longitudinal section of an abdominal spiracle of a third instar larva, showing tracheae entering the atrium beneath the spiracular plate and bulla. The plane of the section is across the crescent of the spiracular plate and through the bulla (see Fig. 23). 250x.

Fig. 27. Transverse section of an abdominal spiracle of a third instar larva. One of the struts supporting the spiracular plate can be seen on the right. The plane of the section is along the crescent of the spiracular plate and through the bulla (see Fig. 23). 250x.

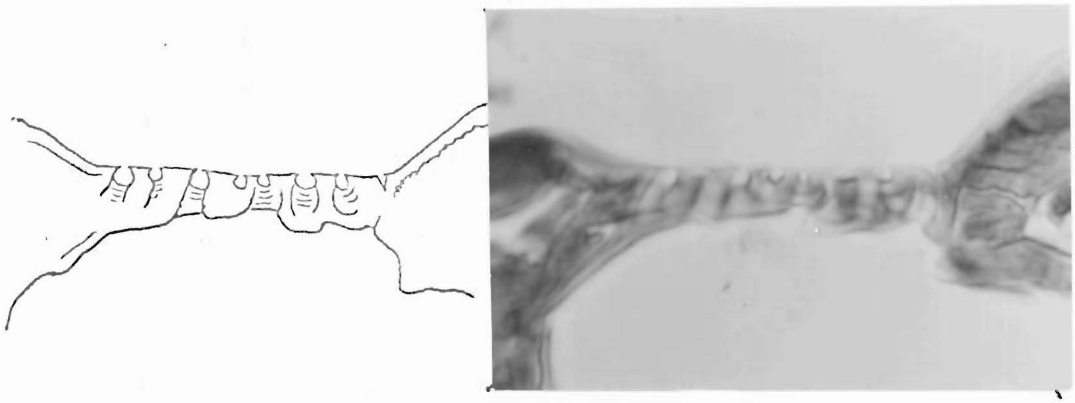


Fig. 28. Enlargement of the spiracular plate of the spiracle shown in Fig. 26. The structure of the aeropyle openings is beyond the resolution of the light microscope; compare the SEM photograph in Fig. 24. 1,250x.

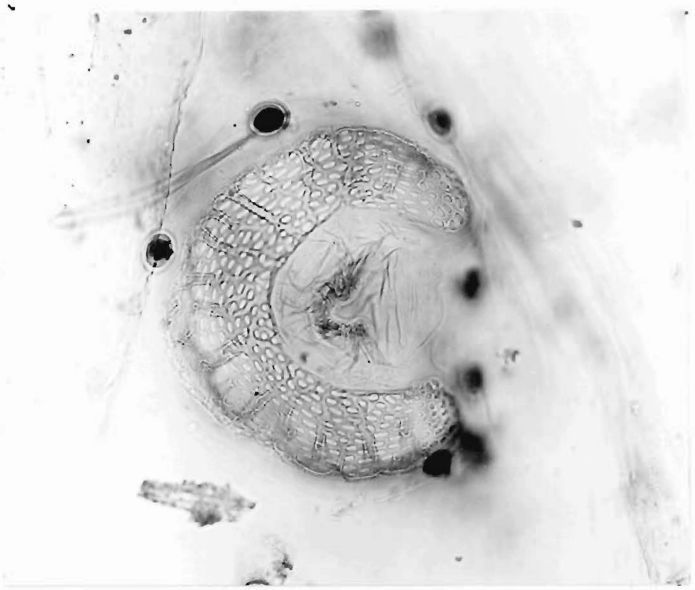


Fig. 29. Cleared whole mount of abdominal spiracle of third instar larva, showing sieve-like spiracular plate. 250x. The dark spots around the spiracle are the 'chitinized discs' (see Fig. 54).

abdominal spiracles. The round button-like structures near the spiracle in fig. 23 appear to be sense organs, and are present around all the spiracles.

Aeropyles in the spiracular plate are shown at higher magnifications in figs 24 and 25. Their lighter edges are artifacts (the sharp edges build up greater charges under the electron beam). Measurements from the photographs show that these aeropyles are up to 0.2 μm across and 5 μm long, similar to those Hinton (1967) found by SEM examination in spiracles of the melolonthid Lepidoderma albohirtum.

Longitudinal and transverse sections of abdominal spiracles (figs 26 and 27 respectively) show that the spiracular plate is supported over an atrium or spiracular chamber by a framework of struts. The tracheae open into the spiracular chamber. The whole spiracular plate with its struts is sclerotised exocuticle, while the cuticle adjacent to the spiracle, covering the bulla and lining the spiracular chamber is mainly unsclerotised mesocuticle.

The structure of the aeropyles can not be seen very well in sections (fig. 28) but as in the other Melolonthinae which have been studied (Boas, 1893; Hinton, 1967) they appear to be narrowest at the opening and flare out to form a chamber several μm wide just below the surface of the spiracular plate. In cleared whole mounts of the spiracle the chamber below each aeropyle and the framework of struts supporting the spiracular plate give the appearance of a sieve, as seen in fig. 29, thus the spiracular plate has been called the sieve-plate or cribriform plate even when the actual openings of the aeropyles can not be seen under the light microscope.

The sections show that there is no mechanism for closing the spiracles of C. zealandica - a characteristic which Hinton (1967) stated is common to all scarabaeid larvae. Water vapour as well as oxygen and carbon dioxide diffuse through such spiracles continually, as Le Berre and Hawlitzky (1967) showed experimentally in Melolontha larvae.

The spiracles of second instar larvae are similar to those of third instar larvae except that they are about a third their size and have fewer aeropyles. The spiracles of first instar larvae are quite different, and their unsclerotised spiracular plates make them very

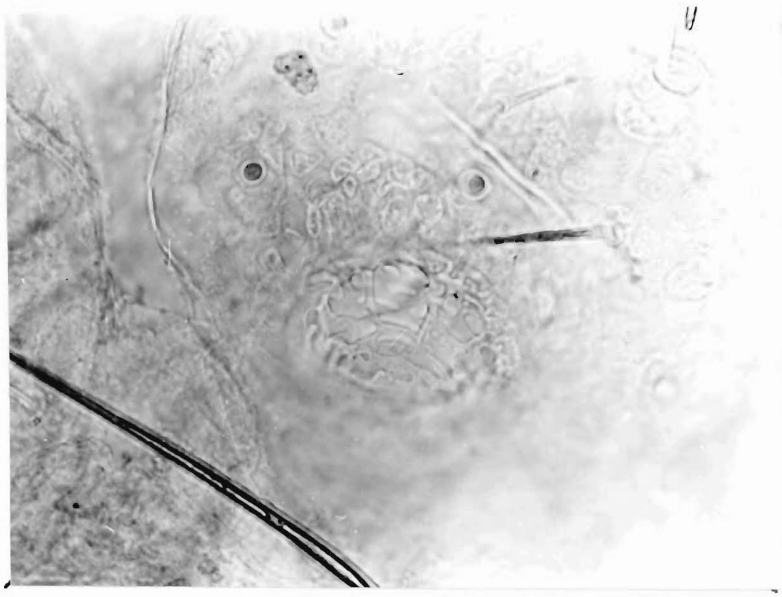


Fig. 30. Whole mount showing spiracular plate (sieve-like structure in centre) of abdominal spiracle of first instar larva. 625x.

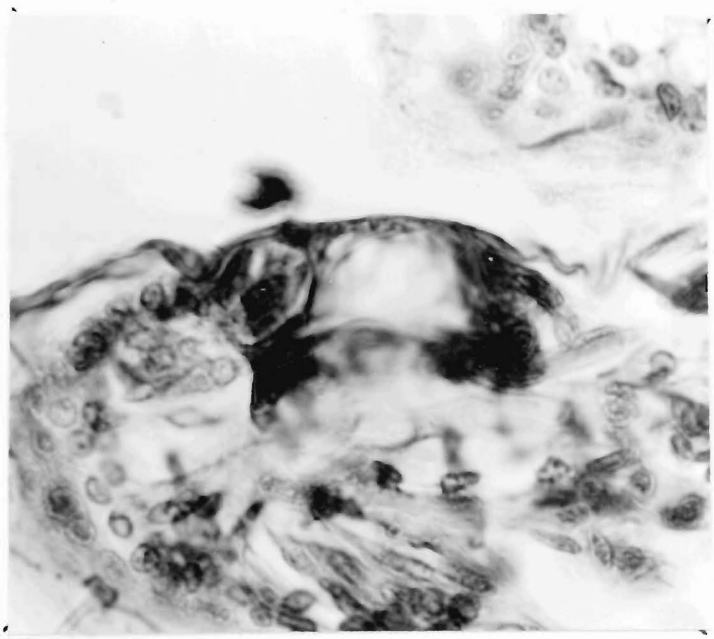
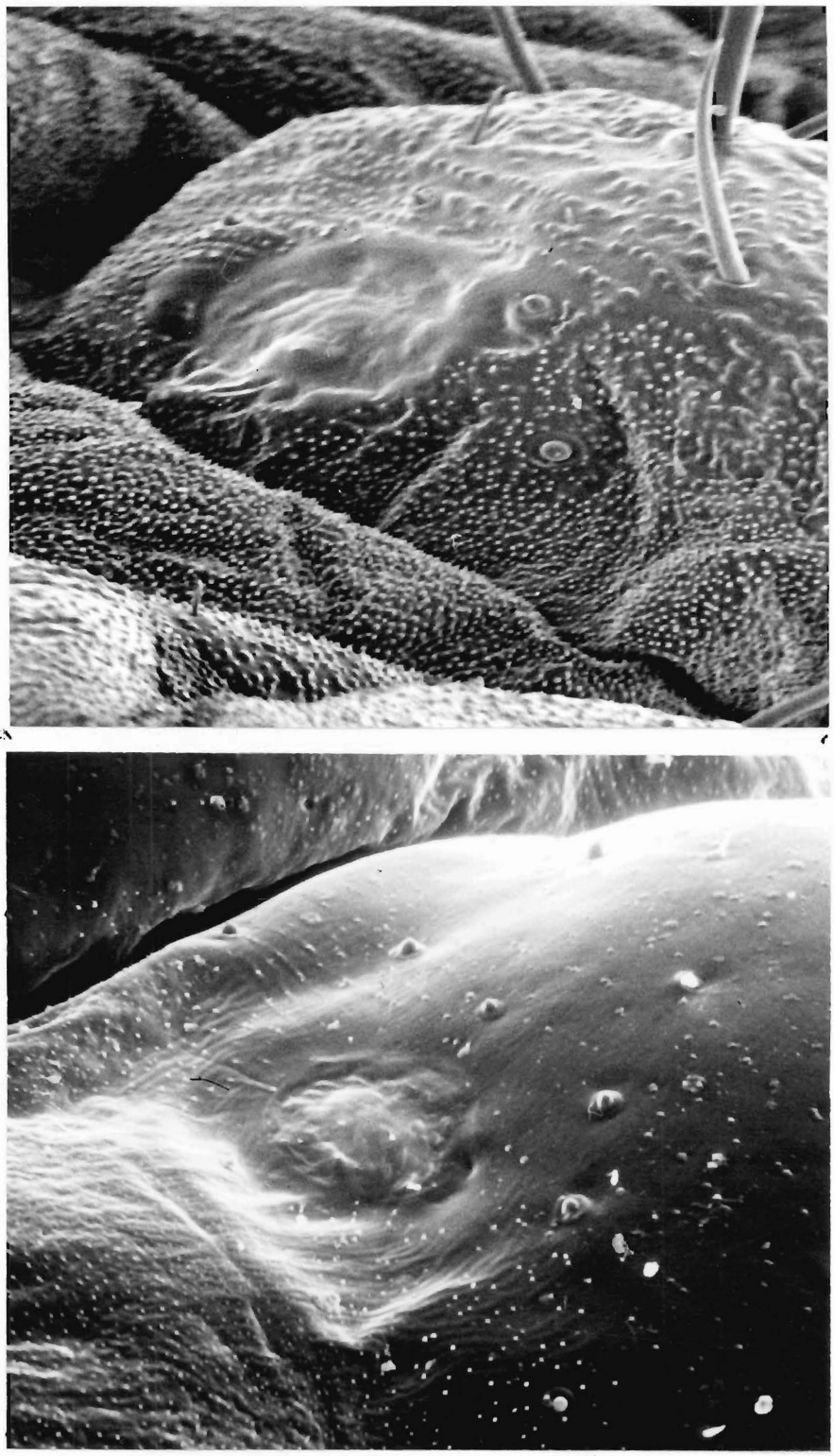


Fig. 31. Section of abdominal spiracle of first instar larva. 750x.



Figs 32 (upper; newly hatched larva) and 33 (lower; older larva).
Abdominal spiracles of first instar larvae. Both 750x.

difficult to distinguish on live animals or in whole mounts in balsam. The spiracular plate, seen in a whole mount in Euparal (a mountant with a lower refractive index) is round instead of crescent shaped, and its struts are asymmetrically arranged (fig. 30).

Figures given by Grandi (1925), Butt (1944) and Lupo (1946) of first instar larvae of other scarabaeid species also show round spiracles. Hinton (1967) stated that in Lepidoderma albohirtum (Melolonthinae) at apolysis "the new spiracle is formed around the lower part of the atrium of the old spiracle". Beginning with the round spiracle of the first instar larva, this mode of formation would produce crescent shaped spiracles of increasing size as are found in the second and third instar larvae.

SEM examination revealed another special characteristic of the spiracles of first instar larvae. On newly hatched first instar larvae the spiracles have no visible aeropyles in their spiracular plates (fig. 32). Larvae approaching the end of the first instar were also examined in case the spiracles of the newly hatched larvae are just temporarily blocked by residues from the egg, and to make sure that aeropyles do not develop during the instar, but their spiracles are the same (fig. 33), with no trace of any openings at all. Sections of spiracles of newly hatched first instar larvae (fig. 31) show the struts below the spiracular plate that were seen in the whole mount (fig. 30), and also the spiracular chamber, but no aeropyles, nor much evidence of tracheae. Thus first instar C. zealandica larvae have closed spiracles, and gas exchanges must take place through their integument.

1.7.2 Surface properties and the effectiveness of the larval spiracles

The larval spiracles must have particular surface properties to be effective, as gas exchanges between the tracheal system and the ambient atmosphere occur by diffusion through the aeropyles in the spiracular plate (Hinton, 1967). A third instar C. zealandica larva has one to two hundred of these fine slits in each spiracle, each about 5 μm long and only 0.2 μm across. Capillary effects occur when water penetrates pores of such small dimensions because of the curvature imposed on the water meniscus. This increases the pressure

on the concave side of the meniscus by Δp:

$$\Delta p = \gamma\left(\frac{1}{R_1} + \frac{1}{R_2}\right).$$

where γ is the surface tension of the water and R_1, R_2 are the radii of curvature of the meniscus. If the pore is circular these radii are equal and then

$$R = \frac{r}{\cos\theta}$$

where r is the radius of the pore and θ is the contact angle between the water and the surface of the pore.

Thus $$\Delta p = \frac{2\gamma\cos\theta}{r}$$ (Adam, 1930).

In an elliptical pore with axes $2r_1$ and $2r_2$ the pressure difference is approximately

$$\Delta p = \gamma\cos\theta\left(\frac{1}{r_1} + \frac{1}{r_2}\right)$$

This pressure therefore forces water into pores with contact angles less than 90° ($\cos\theta$ positive) or out of pores with contact angles greater than 90° ($\cos\theta$ negative). Hence if the advancing contact angle on the spiracular plate and its aeropyles is <u>below</u> 90°, liquid water is drawn into the aeropyles, and may also condense into them from atmospheres of high relative humidity (Briggs, 1967). If the receding contact angle is <u>above</u> 90°, water is forced out of any aeropyles that become flooded.

The diffusion coefficient for oxygen diffusing through water (Kohn, 1965) is about 10,000 times smaller than that for oxygen diffusing through air (Meidner and Mansfield, 1968). Thus diffusion of oxygen through spiracles flooded with water would probably not be enough for the metabolic requirements of the second or third instar larva, although in the first instar larva, which has no aeropyles in its spiracles, diffusion through the cuticle apparently suffices.

The spiracular plates of second and third instar <u>C. zealandica</u> larvae are sclerotised and thus may have contact angles similar to those of the sclerotised head capsule: 102° (advancing) and 89.5° (receding). With these angles the risk of the spiracles flooding would not be great. However, instead of measuring contact angles on the tiny spiracular plate, the resistance of the spiracle to flooding

can be found directly by measuring the pressure required to force water through the aeropyles into the tracheal system.

The method used was based on the technique of Hagmann (1940) for injecting a dye solution into the tracheal system of an insect. A solution of Rhodamine B in distilled water was used instead of the dye solution used by Hagmann (which contained detergent), and the insects were killed with cyanide instead of chloroform (which might affect the surface properties of the spiracles). Larvae were suspended above the dye solution in a flask and the pressure reduced to a predetermined level measured by a Mercury manometer. Then the larvae were shaken down into the dye solution and left for five minutes before the pressure was raised slowly to atmospheric, thus exerting a known pressure on the spiracles of the larvae immersed in the solution (assuming the volume of the tracheal system did not change). The larvae were left in the dye solution for a further five minutes then washed quickly and examined to see whether the dye had penetrated into the tracheae branching from the spiracles which can be clearly seen through the cuticle.

By repeating this procedure with different pressures it was found that few spiracles were penetrated by the dye solution under pressures below 250 mm Mercury (0.33×10^5 N m^{-2}). The surface tension of the dye solution (calculated from its rise in a glass capillary) was 45 dyn cm^{-1} hence from the equation derived above, the contact angle in the aeropyles corresponding to this pressure is 94.1°, which is within the range measured on sclerotised cuticle on the larval head capsule.

Any surface active agent which lowers the contact angle in the aeropyles below 90° should cause them to fill without any applied pressure. This was confirmed by repeating the experiment with detergent ('Teepol') added to the dye solution. Larvae immersed in clean water remained active for up to a week but larvae dipped into water containing detergent were immobilised within a few minutes - confirming the prediction that oxygen diffusion would be insufficient when the spiracles were flooded. Hence the surface properties of spiracles of the larvae ensure that they function effectively in

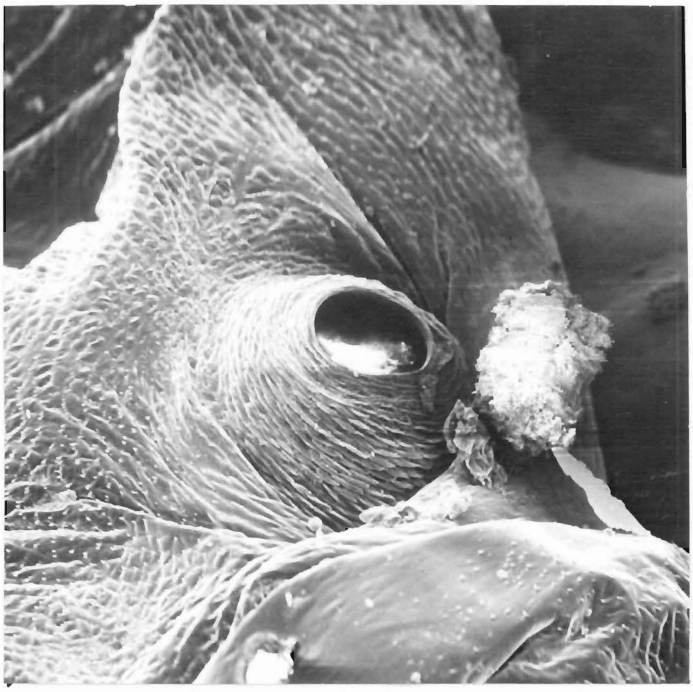


Fig. 34. SEM photograph of second abdominal spiracle of pupa. 220x.

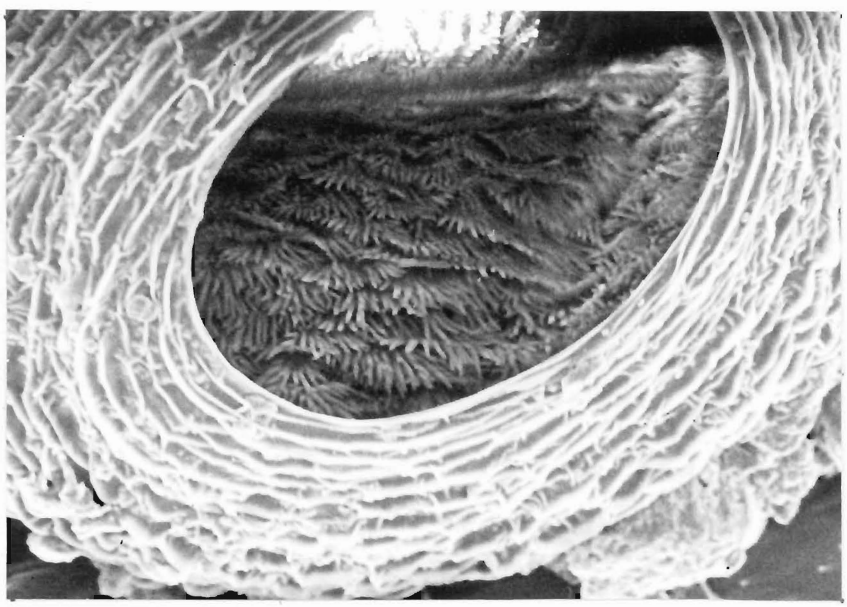


Fig. 35. SEM photograph of filter apparatus of abdominal spiracle of pupa, seen through the spiracular opening. 800x.

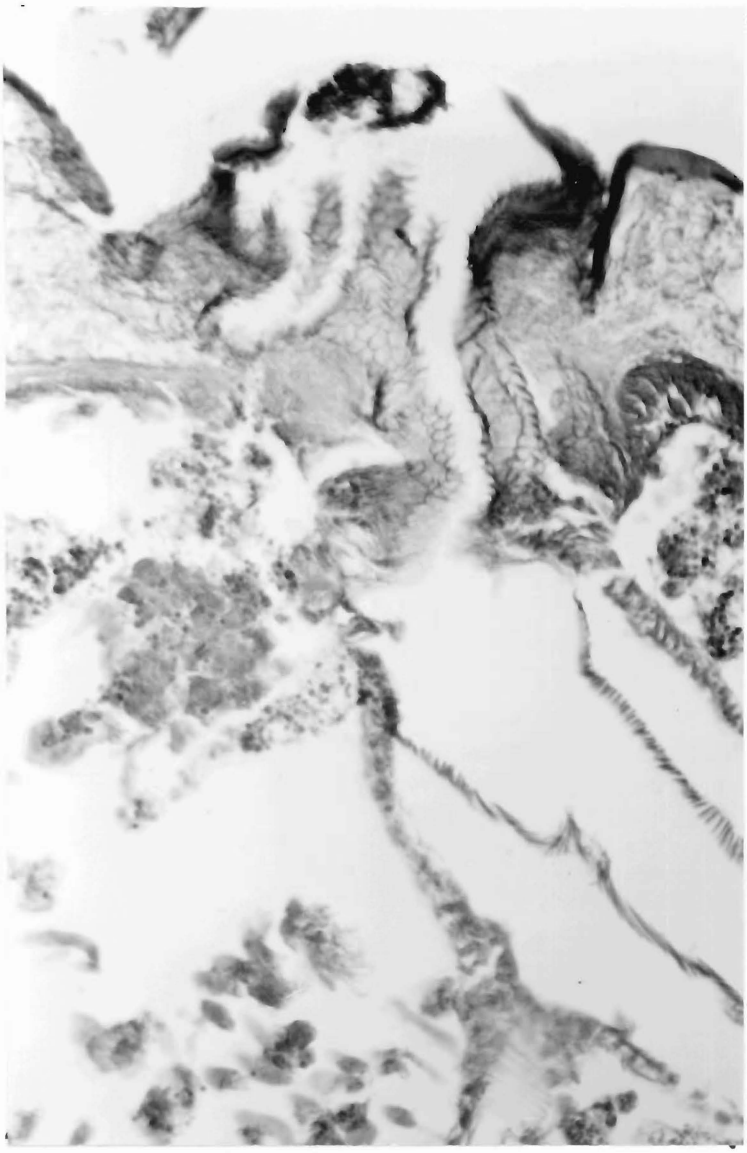
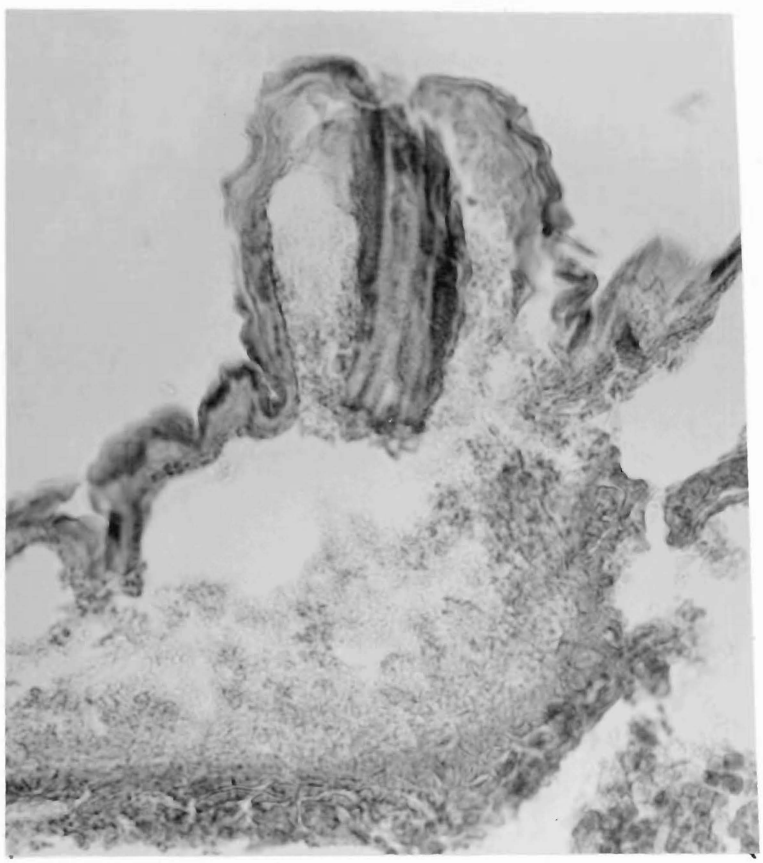


Fig. 36. Section of abdominal spiracle of pupa and underlying adult spiracle. 250x.



Figs 37 (upper; SEM photograph) and 38 (lower; section).
Rudimentary fifth abdominal spiracle of pupa. Both 520x.

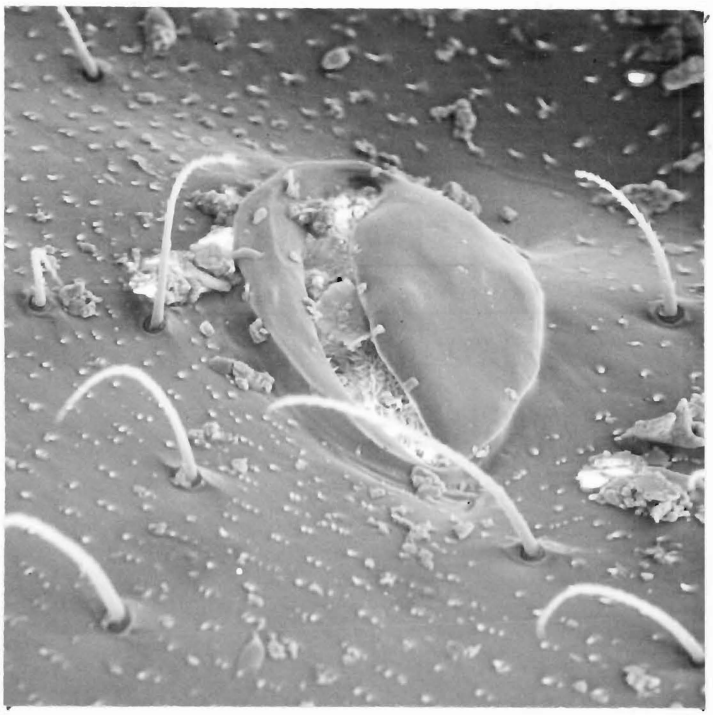


Fig. 39. SEM photograph of an abdominal spiracle of an adult beetle. 520x.

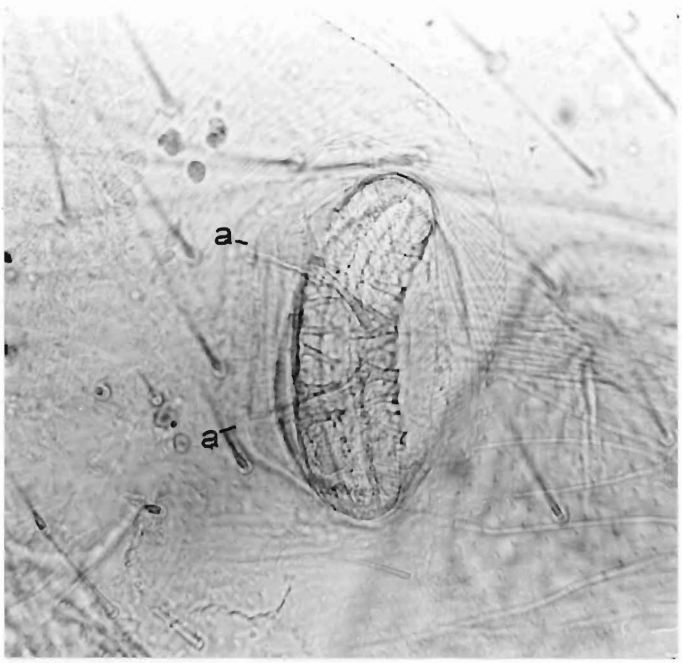


Fig. 40. Light microscope photograph of an abdominal spiracle of an adult beetle. The closing mechanism is visible as two apodemes (a) with an occlusor muscle between them. 250x.

normal conditions but surface active agents make them vulnerable to flooding.

1.7.3 Morphology of the pupal spiracles

The pupa also has nine pairs of spiracles. Those on the prothoracic segment and the first four abdominal segments have a sclerotised outer lip in the form of a truncated hollow cone, which can be seen in the SEM photograph of the second abdominal spiracle in fig. 34. Sections of such spiracles show that a developing adult spiracle lies close beneath each one (fig. 36), so that the pupal spiracles are connected to the tracheal system of the adult, as in other insects (Hinton, 1968). The channel joining the two is guarded by a network of fine branching spines, also seen in the SEM photograph in fig. 35. Hinton (1966) called this type of structure a 'felt chamber', which "reduces to insignificance the tidal flow of air through the spiracles". These spiracles are apparently functional, but the remaining ones on abdominal segments five to eight are rudimentary and in SEM photographs appear to be closed (fig. 37). Sections confirm this (fig. 38) and show that unlike the others, the rudimentary spiracles are not associated with an adult spiracle. Hinton (1966) noted that such spiracles are 'non-functional' only in gas exchange; when they are formed they "provide a lumen through which the old tracheae of the previous instar may be withdrawn."

1.7.4 Morphology of the adult spiracles

Adult C. zealandica beetles have small inconspicuous spiracles and only seven abdominal pairs were found. Ritcher (1969) reviewed the structure of adult abdominal spiracles of the Superfamily Scarabaeoidea; his terms are used in the following description.

The structure of the adult spiracle is quite different from that of the larval or pupal spiracles. The external structure can be seen in the SEM photograph in fig. 39. A sclerotised plate (the peritreme) surrounds the elongated spiracular opening, through which a dense network of fine branching spines like the felt chamber of the pupal spiracle can be seen. Ritcher (1969) called this the filter apparatus. Because of the toughness of the cuticle, the spiracles distorted when sections were cut. The section in fig. 41 shows the peritreme and

Fig. 41. Section of an abdominal spiracle of an adult beetle showing the filter apparatus. 375x.

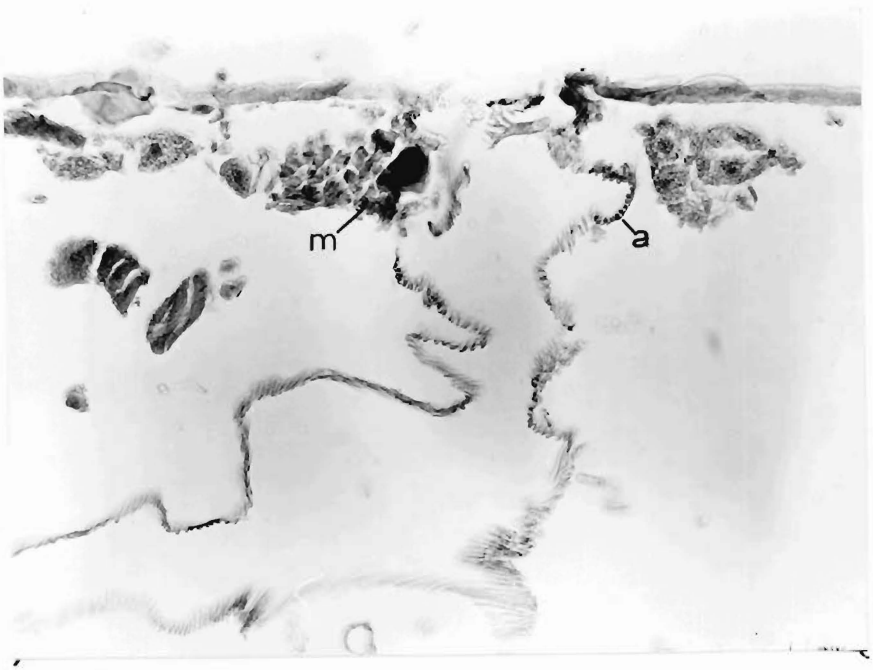


Fig. 42. Section of an abdominal spiracle of an adult beetle showing the associated trachea, and the closing mechanism: the occlusor muscle (m) on the left of the atrium of the spiracle and the thickened atrial wall (a) on the right. 250x.

filter apparatus, which is carried by branches or trabeculae arising from the inner walls of the peritreme. The space within the spiracle is divided by the filter apparatus into an upper atrium and a lower subatrium. At the junction between the subatrium and the large spirally thickened trachea is the closing mechanism, seen in cross section in fig. 42 as a thickening of the wall of the right side of the atrium and an occlusor muscle across the left side. The closing mechanism is also seen in the cleared whole mount of a spiracle in fig. 40 as two subatrial apodemes projecting from the side of the atrium, with the occlusor muscle attached between them. The action of this muscle was not studied, but Ritcher (1969) stated that "when the muscle contracts, the tracheal opening is closed." Fig. 40 also shows the arrangement of the trabeculae branching from within the peritreme. In all details, the adult spiracle of C. zealandica is no different from those described by Ritcher (1969) from other Melolonthinae.

The adult beetle, the only stage with a mechanism for closing its spiracles, is also the only stage which normally emerges into the open air. However, as the transpiration rate experiment showed, it still loses water by evaporation in dry air quite rapidly.

1.8 Morphology of the sense organs

The active larval and adult stages of C. zealandica are capable of perceiving variations in some properties of their environment, as will be demonstrated. They were examined by SEM and light microscope for possible sense organs, such as those found by Jepsen (1937) on larvae of Serica brunnea (Melolonthinae) and by Lupo (1946) on larvae and adults of Anomala ausonia (Rutelinae). The antennae were examined with special care since hygroreceptors (organs sensitive to variations in humidity) are present on the antennae of many species of Coleoptera (Roth and Willis, 1951).

1.8.1 Sense organs on the larval antennae

A variety of structures believed to be sense organs were found on the antennae of the larvae, mainly on the last of their four segments. Four types can be seen in the whole mount of the tip of an antenna of

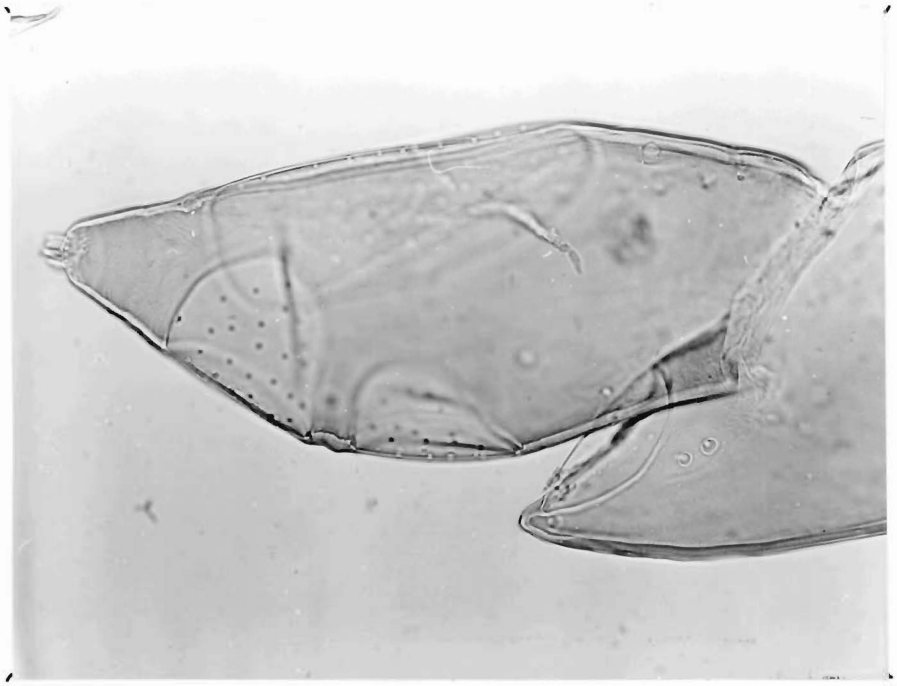


Fig. 43. Light microscope photograph of fourth and part of third segments of larval antenna. 250x.

Fig. 44. SEM photograph of papillae at the tip of a larval antenna. 2,200x.

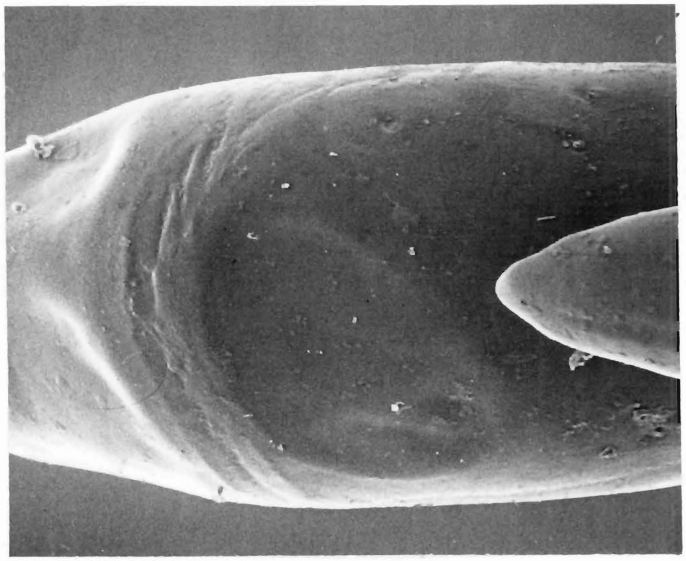


Fig. 45. SEM photograph of the last segment of a larval antenna showing a membranous area (oval depression in centre). 500x.

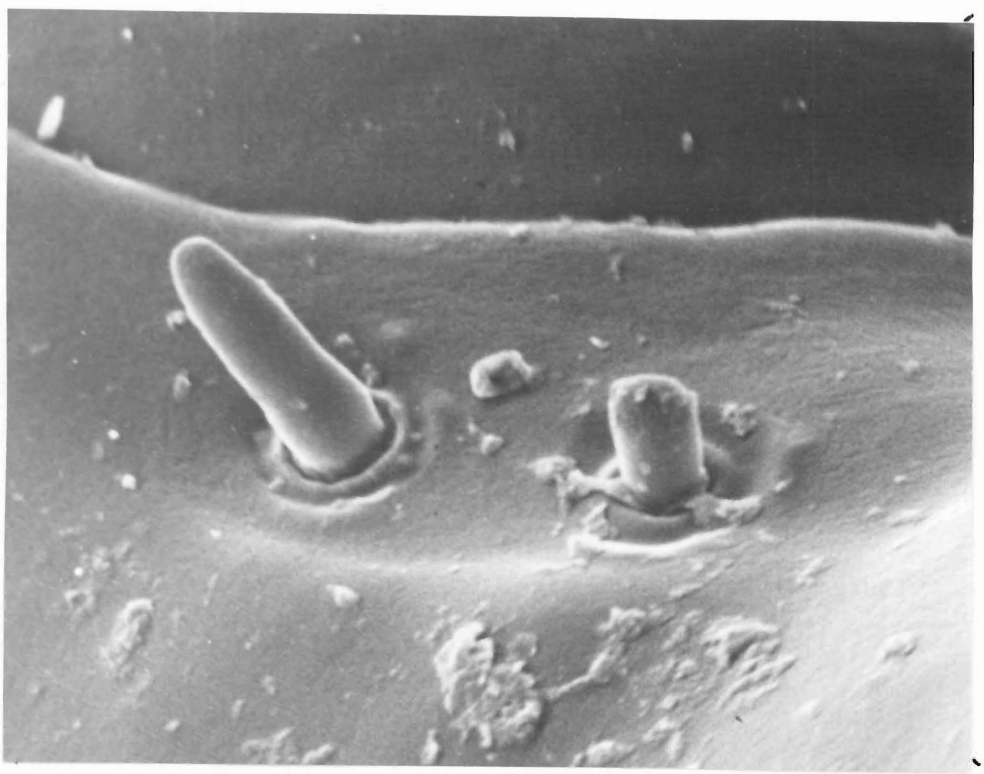


Fig. 46. SEM photograph of a pair of 'chitinous sense pegs' on the third segment of the larval antenna. 5,000x.

Fig. 47. SEM photograph of antenna of adult beetle. 45x.

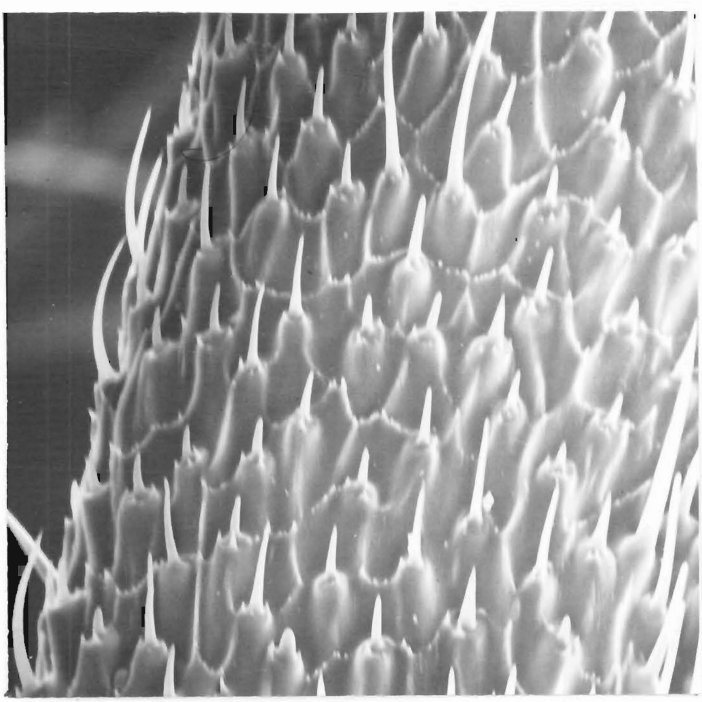


Fig. 48. SEM photograph of spines on a lamella of the adult antenna. 950x.

a third instar larva (cleaned in potassium hydroxide) which is shown in fig. 43:

1. A group of papillae at the tip of the last segment. Sense organs like these on the antennae of the larva of the scarabaeid Cotinis nitida were called "olfactory pegs" by McIndoo (1931). The SEM photograph in fig. 44 shows that there are nine papillae in a ring surrounding two more papillae and a longer spine or hair. This arrangement of apical papillae is also found on the antennae of first and second instar larvae.
2. Three membranous aréas (with thickened spots) on the last segment, and a similar area (without the spots) on the inside face of the extension of the third segment. McIndoo (1931) called this type of sense organ the "compound olfactory organ". These areas appear only as slight depressions in SEM photographs (fig. 45) with no trace of the thickened spots, which must therefore be below the surface of the cuticle.
3. Four pegs near the edges of the membranous area on the third segment. Jepsen (1937) described these as "chitinous sense pegs". The pair clearly visible in the whole mount in fig. 43 are shown in an SEM photograph in fig. 46.
4. Isolated round thickenings in the cuticle, scattered over all four segments. These correspond to the "innervated pit, visible externally as a minute bordered pore" of Jepsen (1937) or the "single olfactory organ" of McIndoo (1931). No trace of these was seen in SEM photographs.

1.8.2 Sense organs on the adult antennae

Given (1952) described the eight-segmented antenna of the C. zealandica adult beetle (fig. 47) and noted that "the last three segments form the club which is not densely pilose, but supplied with placoid sensillae". Round cuticular structures like placoid sense organs can be seen in whole mounts of the antennae but close examination shows a fine curved unpigmented spine arising from each one. The SEM photograph in fig. 48 shows these small spines and the larger spines scattered among them. These two types correspond to the "articulated projections very like hairs...beneath each of which

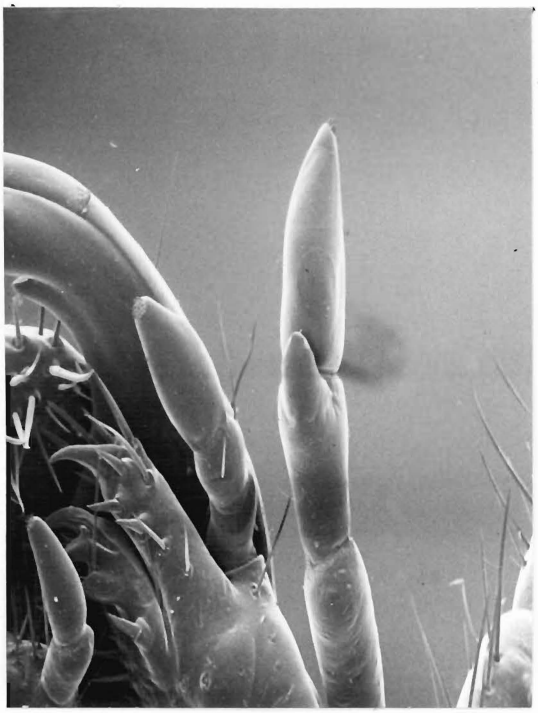


Fig. 49. SEM photograph of mouthparts of first instar larva, showing labial and maxillary palps and antenna. 100x.

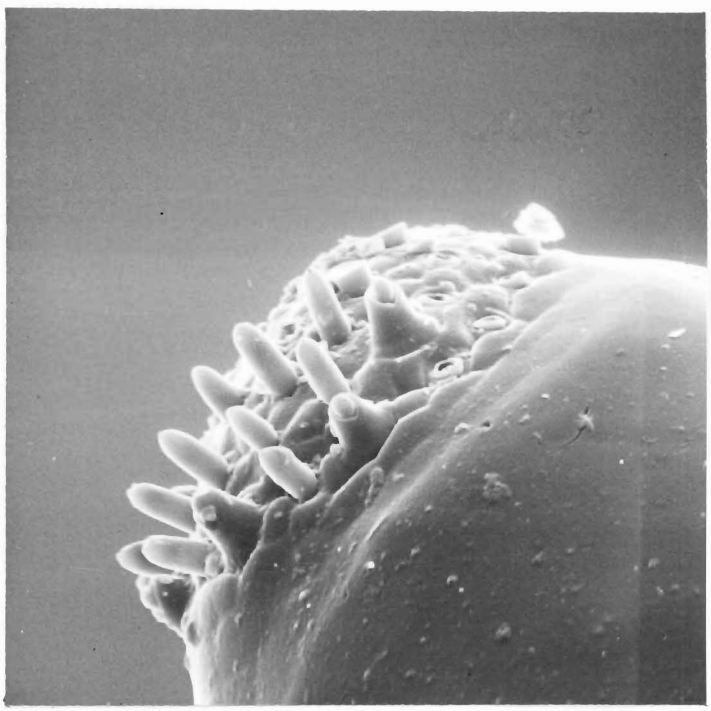


Fig. 50. SEM photograph of papillae on the tip of the maxillary palp of an adult beetle. 1,900x.

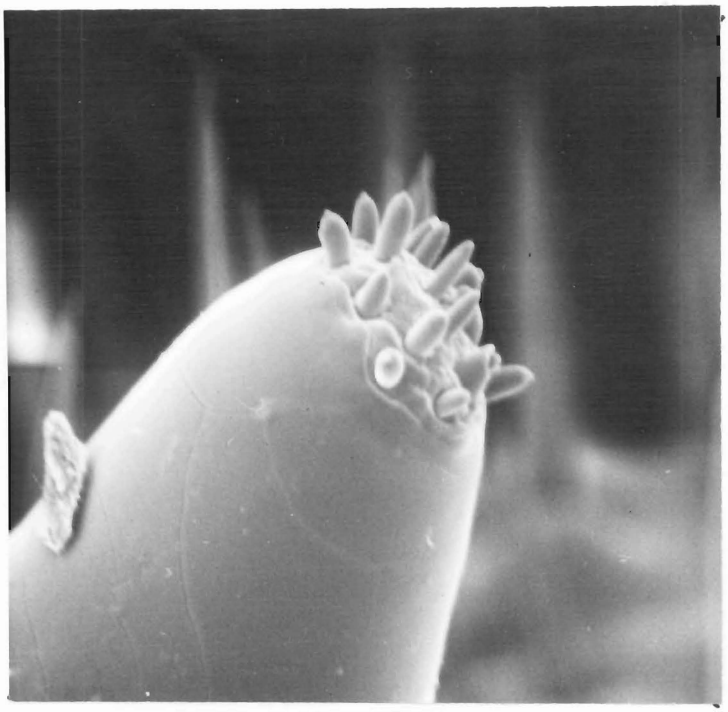


Fig. 51. SEM photograph of papillae on the tip of the labial palp of a first instar larva. 2,100x.

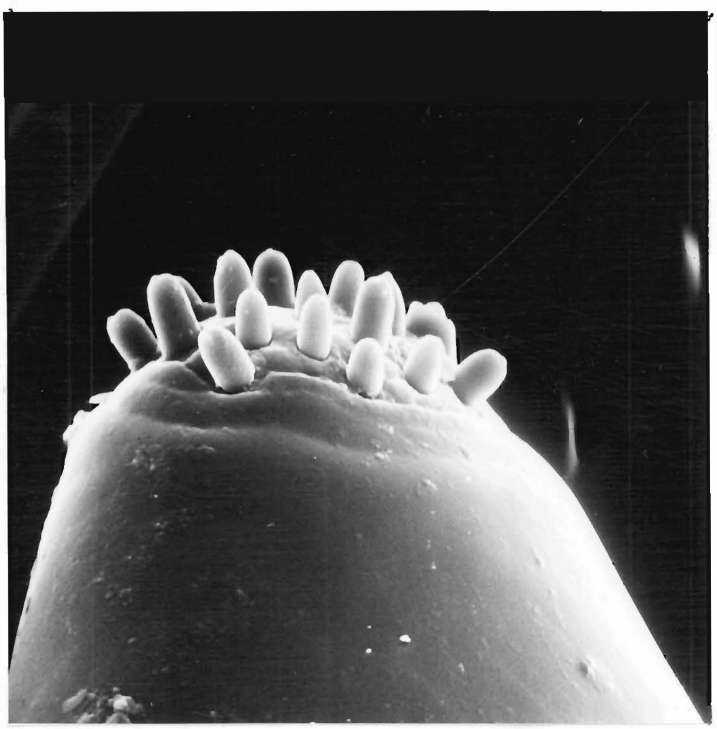


Fig. 52. SEM photograph of papillae on the tip of the labial palp of an adult beetle. 2,200x.

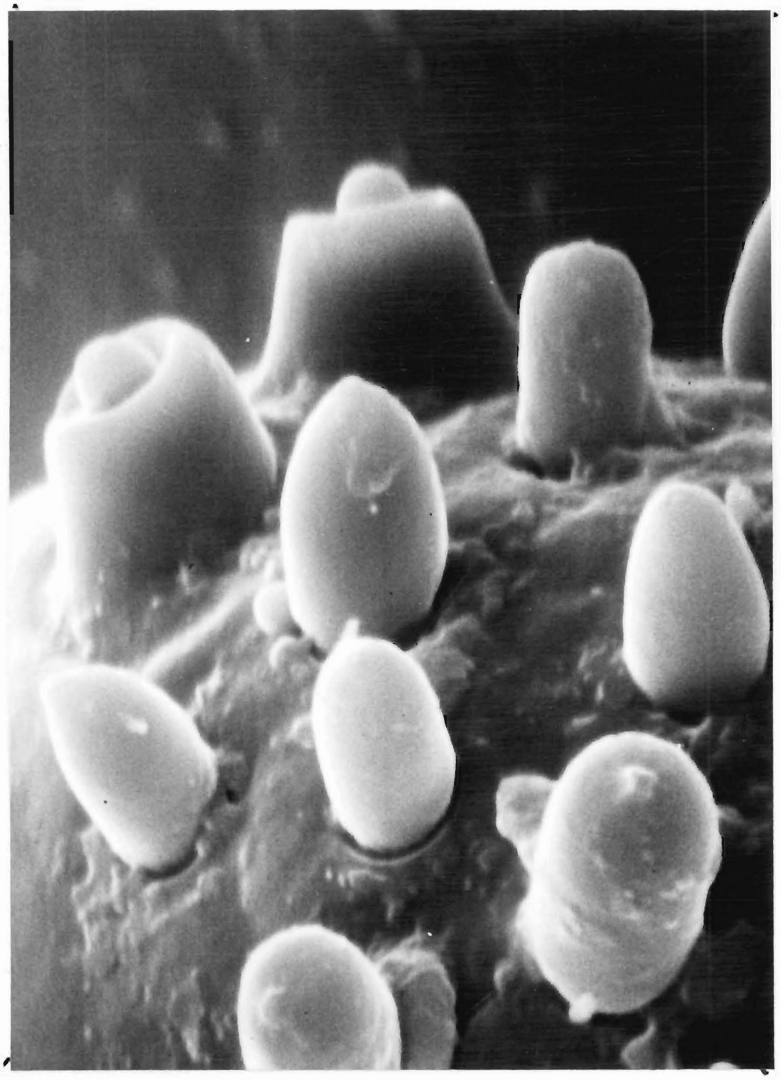


Fig. 53. SEM photograph of papillae on the tip of the labial palp of an adult beetle. 9,000x.

is a sac" and the "true hairs" that Hicks (1859) found on the antennae of the scarabaeid Geotrupes stercorarius. Sense organs on the antennae of Popilius disjunctus (Scarabaeoidea; Passalidae), similar to the fine spines, were shown by Slifer and Sekhon (1964) to be 'thin-walled peg' chemoreceptors. Potassium hydroxide treatment destroys this type of sense organ (Slifer, 1970), which may explain why Given (1952) did not see them. Lupo (1946) described the antennae of the scarabaeid Anomala ausonia as being covered with small round pits, but he treated his material with potassium hydroxide.

1.8.3 Sense organs on the labial and maxillary palps

The labium and maxillae of the larval and adult instars of C. zealandica bear segmented palps (fig. 49), each with a group of papillae at its tip. Lupo (1946) figured similar structures on the palps of Anomala ausonia. There are about 15 papillae on the labial palps (figs 51 and 52) and about 25 on the maxillary palp (fig. 50). Their size and number is the same in all the active instars, from the first instar larva (fig. 51) to the adult beetle (fig. 52). Each papilla is about 3.5 μm high and 2 μm across, and where papillae have broken off during preparation of the animals for the SEM, walls about 0.6 μm thick can be seen (fig. 50). The significance of the smooth sheaths around some of the papillae (fig. 53) is not known.

1.8.4 Other sense organs

Ritcher (1966) characterised larvae of the tribe Sericini of Melolonthinae by the "groups of dark granules present on the cardo and articulating membrane of the maxilla, on the prothoracic shield, below the spiracles, on the coxae of the legs, on the sclerites adjacent to the coxae, and elsewhere on the body". These 'dark granules' are the sense organs described as 'chitinized discs' by Jepsen (1937), who also stated that they were characteristic of Sericini. SEM examination of C. zealandica larvae revealed these sense organs in the areas listed above. Those near the spiracles, which have already been mentioned, have a flattened dome-shaped centre about 10 μm across with a finely textured surface, and are set in a depression with slightly raised edges (fig. 54). A similar sense

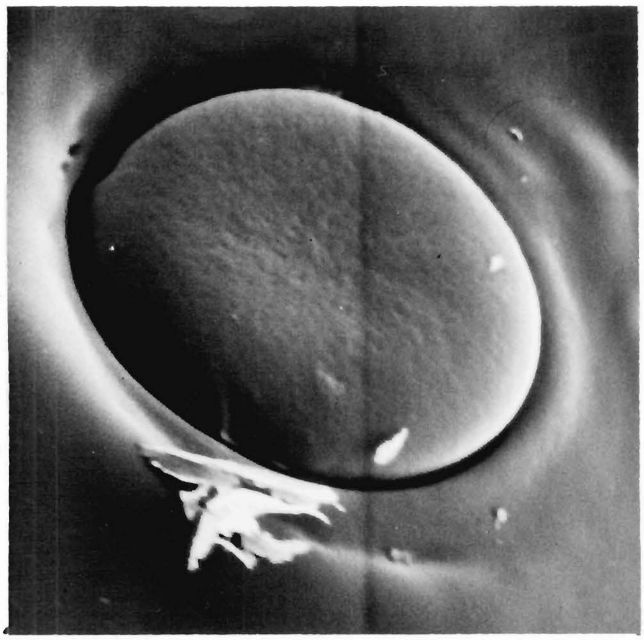


Fig. 54. SEM photograph of a 'chitinized disc' near an abdominal spiracle of a third instar larva. 6,000x.

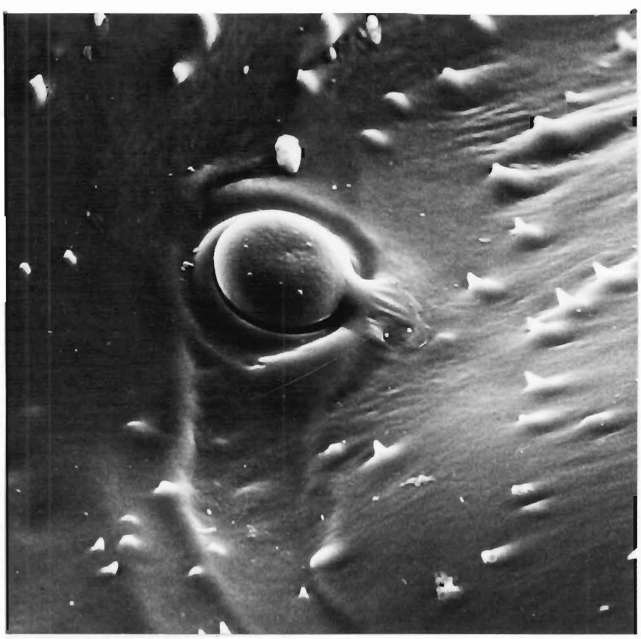


Fig. 55. SEM photograph of a 'chitinized disc' on the thorax of a third instar larva. 2,400x.

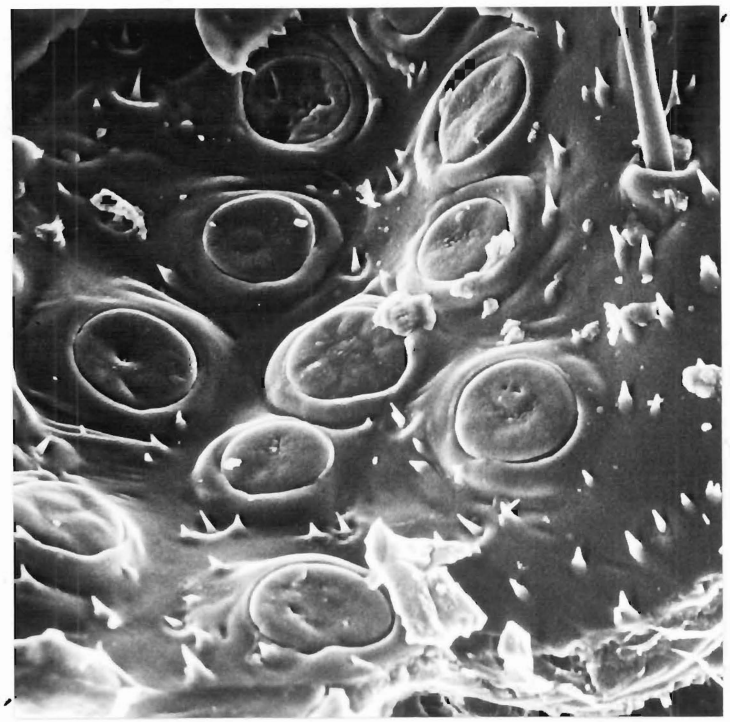


Fig. 56. SEM photograph of the 'chitinized discs' on the maxilla of a third instar larva. 1,000x.

organ photographed on the thorax of a third instar larva (fig. 55) is only 6 µm across, and appears to have a 'bridge' across to the dome, but this may be contamination or secretion from the organ. The sense organs crowded together on the dorsal face of the cardo of the maxilla (fig. 56) are rather different. The flattened dome or disc is about 15 µm across and sometimes has a cellular pattern on its surface. The thickening of the cuticle around the disc is more prominent than in the other similar sense organs.

The range of structures found which appear to be sense organs suggests that C. zealandica can perceive a wide range of stimuli but no specific sensory function has been associated with any of them. Using electrophysiological techniques, Pham-Binh-Quen (1969) "discovered olfactory receptors at the ends of the antennae which were capable of reacting both to dissolved and to gaseous stimuli" and "taste receptors...on the maxillae and labrum" of Anisoplia austriaca (Melolonthinae) larvae, but gave no details of the types of sensillae involved.

2. The environment

2.1 Introduction

The normal environment of C. zealandica is soil and only the adult beetle normally emerges above ground. Soil is a specialised environment with some features of aquatic as well as normal terrestrial environments.

The particles of various sizes which make up the solid matrix of the soil are usually clustered into aggregates. The pores between the particles are occupied by soil water containing dissolved material, and soil air which may have a different composition from atmospheric air. Animals and plants live in the pores and on the surfaces of the soil particles.

Like any porous material, soil retains by surface tension and other forces, some water which does not drain under the action of gravity. The force required to remove water held by surface tension depends on the curvature of the air-water interface in the pores of

the soil. This curvature is determined by the size of the pores, so as the amount of water in the soil decreases the water retreats into smaller and smaller pores and the force required to remove it increases. This applies to biological as well as physical agents working to remove water from the soil, so the concept of a force holding the water in soil provides a possible way of expressing the water stress on an animal in the soil in more definite terms.

Water evaporates more and more slowly from soil as it dries because the path through the solid matrix available for diffusion of water vapour out of the soil is tortuous and small in area. Thus with rain occasionally replenishing the water in the soil, and special properties limiting the rate of loss, enough water normally remains in the soil to keep the humidity in its air spaces near saturation. This study is concerned with the consequences of such a moisture regime on the life of C. zealandica; other special features of the soil environment, such as permanent darkness, confined and abrasive habitat, periodic flooding and gas concentrations differing from normal atmospheric proportions (Rapoport and Tschapek, 1967), are considered only where they affect the main issue.

2.2 The soil at West Melton

All samples of C. zealandica used in this study were collected from a large population in a field off Adams Road, West Melton, 8.5 miles from Christchurch Airport. An adjacent area was used for a trial of insecticides against C. zealandica by Read (1969). The soil in this area consists of a shallow layer (23 cm) of silt loam (a recent soil of the Eyre-Paparua series) overlying fine sandy loam (Halkett yellow-brown sand) (N.Z. Soil Bureau, 1965). Paparua silt loam closely resembles Templeton silt loam, a reference soil described from a site 7.5 miles away by N.Z. Soil Bureau (1968). All detailed descriptions in this study of the environment of C. zealandica refer to the West Melton site.

The structure of the West Melton soil inhabited by C. zealandica is shown in figs 57-59. Thin sections of the soil were made by the technique of Mitchell (1956) as modified by Langton and Lee (1965), using Araldite to glue the sections to ordinary glass slides.

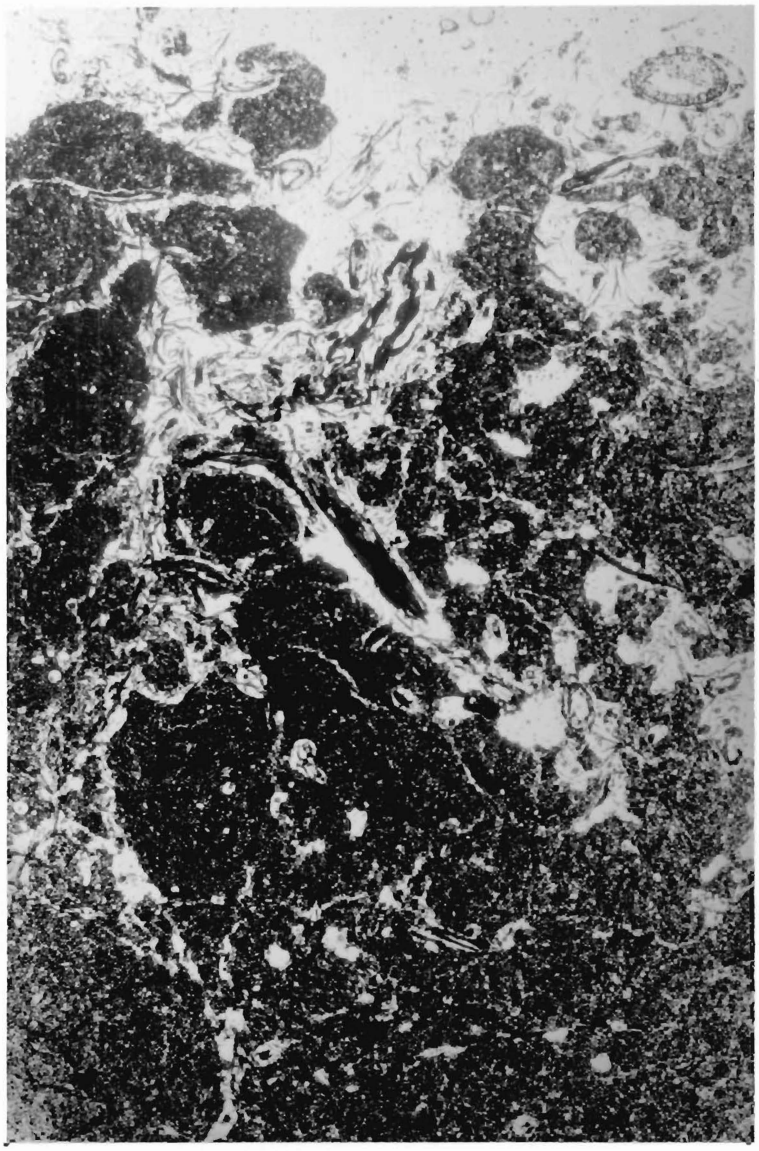


Fig. 57. Thin section showing the structure of the soil near the surface. 5.7x.

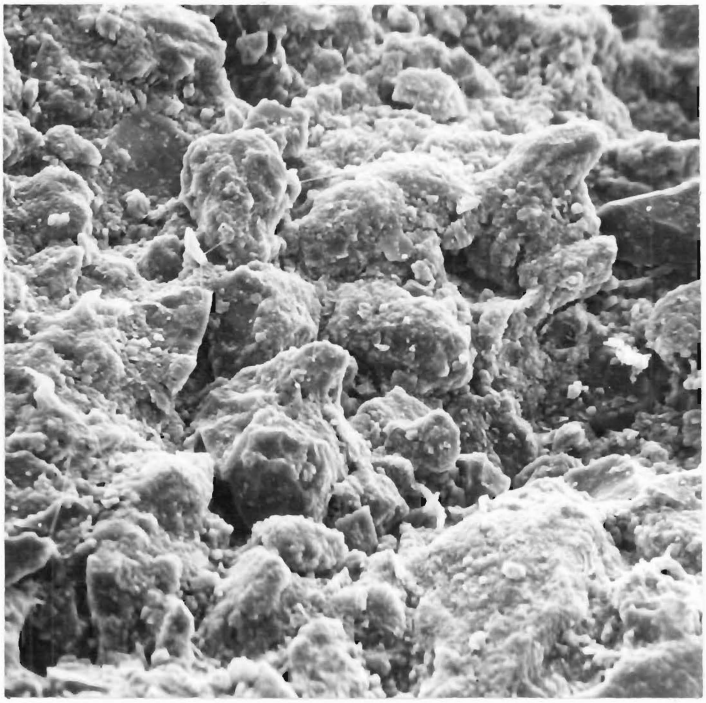


Fig. 58. Soil - an SEM view. 500x.

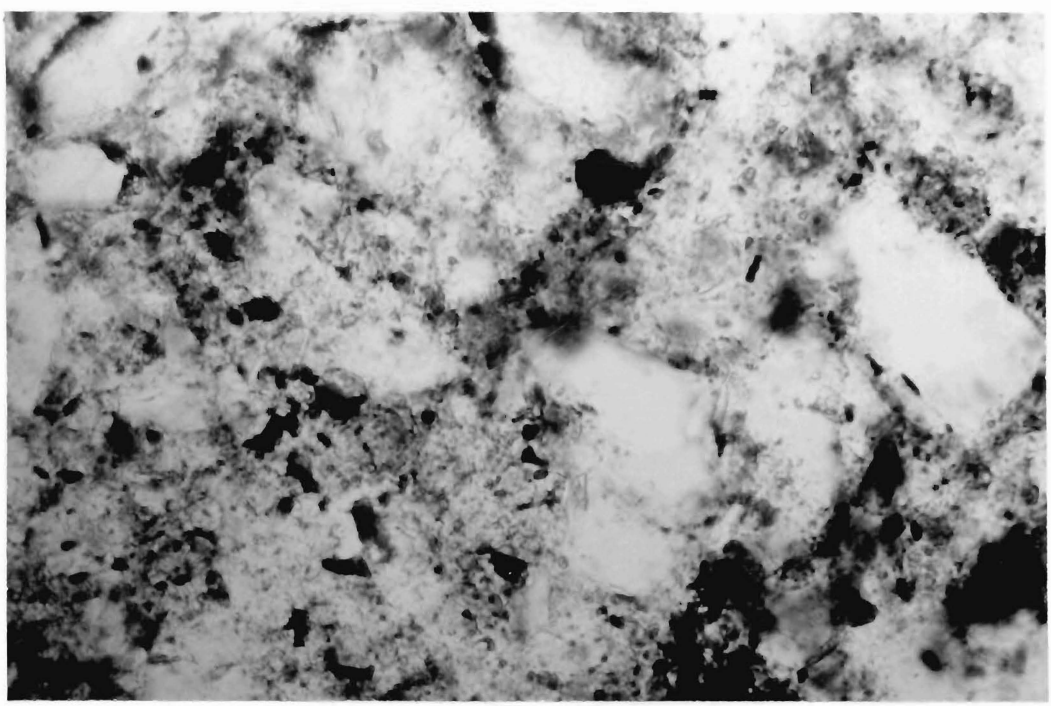


Fig. 59. Soil - as seen in a thin section. 500x.

SEM photographs were taken of the surface of a natural soil clod prepared by freeze drying and coating with metal as before. The section of the upper 2 cm of the soil in fig. 57 shows the aggregated structure of the soil and the abundant plant material in this zone. Finer roots extend much deeper into the soil. Fig. 58, an SEM photograph of the surface of soil, and fig. 59, a photograph of a thin section show the irregular mineral particles of various sizes (sand, silt and clay) and the organic material covering them, that make up the soil.

2.3 The environment of the active larval instars

Within the soil, each stage of C. zealandica has its own particular place. The larvae have a different relationship with the soil environment from the other stages. All stages require water for development but the larva also requires food and must move through the soil to find it.

Animals move through the soil in a variety of ways. Kühnelt (1955) differentiated between burrowing and non-burrowing soil animals. Kevan (1962) subdivided the 'burrowers' into 'excavators' which dig through the soil and 'tunnellers' which squeeze through existing spaces. Among the excavators he included 'miners' which dig with their jaws and 'fossors' or 'diggers' which dig with their legs or other modified parts. Among the tunnellers he included 'soft bodied tunnellers' which burrow by peristaltic movements and 'rigid tunnellers' which push their way through the soil.

C. zealandica larvae were observed burrowing in a thin layer of soil between two sheets of glass held in a wooden frame. The larva normally lies curled in a C shape in a cell in the soil. It extends the cell or burrow by digging into the soil with its mandibles, either holding them together and scraping the soil away, or gnawing at the soil with small movements of the mandibles. During these movements the dorsal surface of the abdomen, which is covered in short spines, is pushed against the soil behind so that the larva can exert leverage with its mandibles. The fragments of soil detached from the wall of the cell are manipulated by the mouthparts, with the

maxillary palps being particularly active, and then raked down by the legs and mouthparts into the space under the abdomen. The digging movements are continued until this space is filled with loose soil. Then the larva tucks its head under and turns to the back of the cell, pushing the loose soil with its head and mandibles. It packs this soil against the wall of the cell with its head, mandibles and legs, and then turns over in the cell again to resume digging at the other end. By digging at the soil around it in this way, the larve forms a closed cell just large enough for it to turn around in. Thus the C. zealandica larva is a 'miner'. Its characteristic mode of burrowing has also been described in other Scarabaeidae (Rittershaus, 1927; Schwerdtfeger, 1939; Gusev and Antonjuk, 1956).

Despite their mobility the larval instars are normally found among "the matted roots of the pasture just below the ground surface" (Dumbleton, 1942). According to Kelsey (1950), "the first stage is not usually found in the top two inches of soil, whereas the second stage is, and the third stage is mainly concentrated in the top one inch of soil". Larvae occasionally move onto the surface of the soil in laboratory experiments (Radcliffe, 1970), but not normally in the field, although Kain (quoted in Radcliffe, 1970) observed them on the surface of pastures with very high larval populations. Other melolonthid larvae move onto the surface in large numbers at night (Malenotti, 1940) so a response to light may be involved. The following experiment was therefore carried out to test whether C. zealandica larvae avoid light.

Larvae were placed, one at a time, in a 'Y' shaped track in a darkroom and a dim light arranged to shine along one arm only of the 'Y', so that when it reached the junction the larva had the choice of crawling into either the light or the dark arm. The light was changed from one arm to the other between each run. Five different larvae chose the dark side 76 times in 100 runs. If a larva was unable to distinguish the light from the dark path it would be expected to choose either at random. This hypothesis must be rejected since the probability of choosing the dark path 76 times in 100 would be much

less than 0.001. The alternative hypothesis is therefore accepted: the larva is able to perceive light and tends to move away from it.

Negative phototactic responses have been demonstrated in other scarabaeid larvae including those of Oryctes rhinoceros (Dynastinae) (Costa and Ganesalingam, 1967) and Rhizotrogus aestivus (Melolonthinae) (Schäfer, 1954). Rudimentary ocelli are usually present dynastine and often in melolonthid larvae, particularly those of the tribe Sericini (Jepsen, 1936; Ritcher, 1966), but no ocelli or other light sensitive organs were found in C. zealandica larvae.

The penetration of light into West Melton soil was also tested, by measuring the attenuation of light passing through sieved air-dry soil packed in a layer less than 1 mm thick over the photocell of a light meter. The light intensity of a lamp fixed directly above the photocell was measured both with and without the soil intervening so that the attenuation of the light during its passage through the soil could be calculated. The thickness of the soil layer was estimated from its area, weight and density. The average attenuation of the light was calculated from several measurements to be about 6.10^9 times per millimeter of soil penetrated. Thus larvae are unlikely to be influenced by light until they are very close to the surface.

Possibly C. zealandica larvae remain near the surface of the soil because they are attracted to plant roots, which are most abundant there. Melolontha larvae in soil are attracted several centimeters to the plant roots they feed on (Klingler, 1957; 1958). Sense organs mediating such a response have also been found, by electrophysiological techniques, on the mouthparts of larvae of the melolonthid Anisoplia austriaca (Pham-Binh-Quen, 1969). A laboratory experiment was designed to find whether second instar C. zealandica larvae congregated near plant roots in soil. Three boxes were filled with soil and sprouting wheat grains placed in one end of each. Twenty larvae were placed on the surface of the soil along the midline of each box. Several combinations of soil moisture were used. The numbers of larvae recovered in each end after one week are given in Table 4.

Table 4. Numbers of C. zealandica larvae recovered near roots in choice experiment.

		With roots	Without roots
Box 1	Soil water content:	24%	12%
	Larvae recovered:	15	5
Box 2	Soil water content:	12%	24%
	Larvae recovered:	14	4
Box 3	Soil water content:	16%	16%
	Larvae recovered:	18	2

The reaction of the larvae to moisture gradients will be discussed later; the effects of the moisture gradients in boxes 1 and 2 are cancelled by grouping these results. There were 47 of the 58 larvae recovered in the ends with roots. Under the null hypothesis that a larva is equally likely to occur in either end of a box the probability of there being as many as this in one end is less than 0.0005. The alternative hypothesis is therefore accepted: the larvae congregated near the roots. In the field such behaviour will concentrate feeding larvae where plant roots are abundant. As the soil sections showed (fig. 57), most of the plant roots are in the top few centimeters of the soil.

The behaviour patterns described all influence the particular environment of the active larval instars. Their mode of burrowing preserves a space about them as they travel through the soil. Since they congregate near roots, but avoid light, most of the feeding larvae remain quite close to the surface of the soil, but rarely move above it.

2.4 Prepupal behaviour and the environment of the pupa

Some time before the third instar C. zealandica larva pupates it ceases feeding and its weight and fat content begin to decline (Perrott, Shorland and Czochanska, 1965). During this prepupal stage the larva burrows down in the soil (Kelsey, 1951), as do other scarabaeid prepupae (Girault and Dodd, 1915). An experiment was carried

out to find whether this is a geotactic response. Round plastic Petri dishes were filled with soil and five prepupal larvae, immobilised by chilling, placed in the soil across the diameter of each dish. The dishes were stacked on edge, so that the larvae were half way down, and kept in darkness to prevent orientation to light. The numbers of live larvae in the top and bottom halves of the dishes counted after 18 hours in one series and after 72 hours in the other were:

	top	bottom
18 hours	12	35
72 hours	14	31

The two distributions are not significantly different ($\chi^2 = 0.35$, $P > 0.25$). Then (combining the two) under the null hypothesis that a larva is equally likely to occur in either half of the dish, the probability of there being 66 or more of the 92 larvae in the bottom half is less than 0.005. The alternative hypothesis is therefore accepted: these prepupal larvae tended to move downwards under the influence of gravity.

During the prepupal phase mature scarabaeid larvae construct cells in which to pupate (Ritcher, 1958). The account of the prepupa of Anomala orientalis by Friend (1927) applies equally to C. zealandica: "The intestine is emptied of its contents and the larva is yellowish white in colour. The abdomen is bent ventrally on itself in a very characteristic position and the legs are folded up at the coxatrochanter joint. Power of locomotion is lost, and the only movement is flexing and reflexing of the abdomen. By this movement a hollow space is formed in the soil, and in this space pupation occurs." Similar cell building behaviour has been described in Anomala ausonia (Lupo, 1949), Melolontha melolontha, Amphimallus solstitialis and Serica brunnea (Fidler, 1936). However, Cumpston (1941) stated that the scarabaeid prepupa "sheds the rectal contents, which are used as a plaster in the formation of the ovoid pupal cell. This plaster ensures a fairly firm and impermeable structure." Hayes (1929) also considered that "the material which holds the

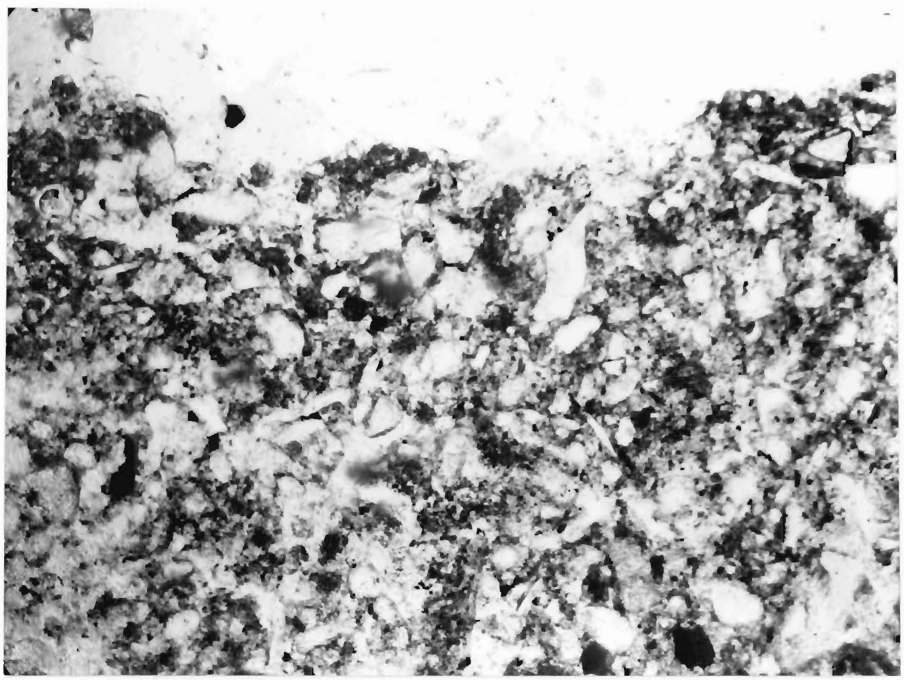


Fig. 60. Thin section showing normal soil structure in the wall of a pupal cell. 100x.

Fig. 61. SEM photograph of the wall of a pupal cell. 100x.

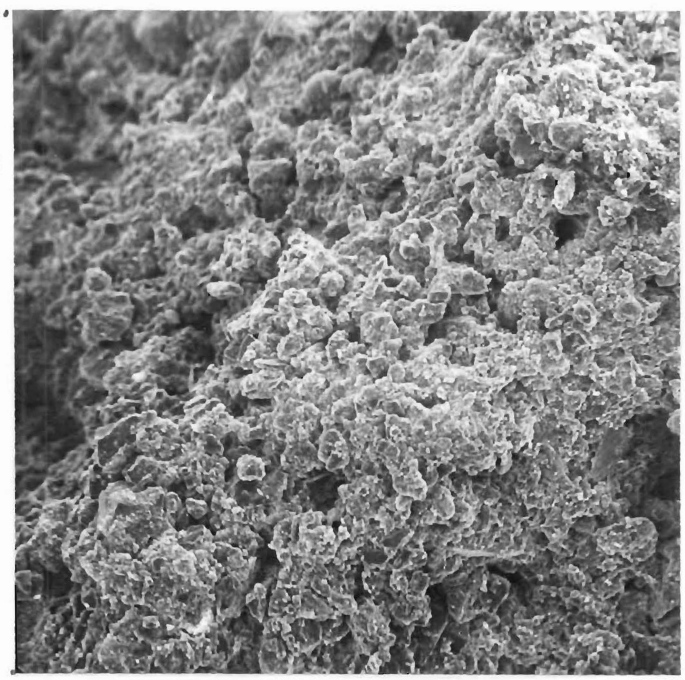


Fig. 62. SEM photograph of the cut surface of the soil adjacent to the pupal cell. 100x.

structure together is a glandular secretion ejected from the hind intestine. The larva, by its mouthparts and body movements, is able to mold the material into a compact water-tight cocoon." Carne and Chinnick (1957) stated that larvae of Sericesthis pruinosa (Melolonthinae) "form cells which they line with their discharged gut contents". On the other hand, Maelzer (1960) could not find any difference between the permeabilities to water vapour of unmodified soil and the wall of the pupal cell of the scarabaeid Aphodius tasmaniae. He concluded that "it is probable...that there is no biological lining of the prepupal cell which influences the rate at which water passes out of the cell."

Prepupal C. zealandica larvae were observed in a thin layer of soil between sheets of glass. A few pupated but no special cell building behaviour was seen. The pupal cell appeared to be modified from the cell made by the normal burrowing of the active larva merely by continued movement of the larva in the same cell leaving the walls of the cell compacted and smooth. Neither sections (fig. 60) nor SEM photographs of the cell wall (fig. 61) show any modification of the soil in the wall, compared with the adjacent soil (fig. 62), beyond mere compaction. There is no trace of any 'plaster' over the surface of the wall. Sections showed that some material remains in the hindgut when it is shed with the larval skin at pupation, thus any spreading of gut contents over the walls of the pupal cell is probably incidental in C. zealandica, and the cell has no special lining.

Ritcher (1939) investigated several species of Scarabaeidae and concluded that "soil structure...and soil moisture may affect the pupation levels but they do not appear to change the relative positions of the various species." At West Melton C. zealandica pupae were found between 6.5 and 15 cm deep in the soil. Kelsey (1950, 1951) found them from 3 to 10 inches (8-25 cm) deep and Miller (1921) at "varying depths of from 1 ft. or more according to the soil". The pupal cells are oval, about 15 mm long and 7 mm across, and are normally aligned horizontally. The shed larval skin

Fig. 63. A pupa in its cell in the soil. 10x.

is pushed to the end of the abdomen of the pupa, as in most Scarabaeidae (Ritcher, 1958), and the pupa lies supine in the cell (fig. 63) incapable of any movement apart from a slow flexing of the abdomen.

2.5 The environment of the adult beetle

The adult C. zealandica beetle remains in the pupal cell until its integument has hardened and then begins to burrow up to the surface of the soil (Miller, 1921). Under Kevan's (1962) classification of burrowing modes, it is a 'fossor', digging with its legs. The fore tibiae are each armed with three strong spines well suited to this task. Where any passage through the soil is available the beetle pushes itself through (the 'rigid tunnelling' mode) aided by its compact shape and smooth hard integument. Mature C. zealandica beetles wait below the surface and then "emerge and fly as twilight wanes" (Thomas, 1913). Apart from the short period of activity at twilight and later in the night they remain in the soil.

2.6 Oviposition and the environment of the egg

The female beetle determines the environment of the egg. Two behaviour patterns seem to be involved: if a female beetle is mated while still on the ground after it first emerges it burrows back into the soil in the same area to lay its eggs, but if it flies before mating it then chooses areas with plant cover rather than bare ground in which to lay its eggs (Power, 1968). The first pattern was observed at West Melton where there was very little bare ground anyway. The second behaviour pattern was tested in a laboratory experiment. To find whether beetles preferred to burrow into bare or grass covered soil, a block of turf from West Melton was cut to fit closely into a box and all plant growth cut off at the soil surface on one half. Beetles picked up from the ground at night at West Melton after a flight were scattered across its surface and the box covered with clear plastic sheet to prevent beetles escaping and to reduce drying of the soil. After a further night the beetles were recovered from the soil. Those on the surface were discarded and the remainder classified by sex and whether they were in the

grass-covered or clear half:

	grass	clear	
male	25	8	
female	102	42	
total	127	50	177

There is no significant difference between the distributions of male and female beetles ($\chi^2 = 0.32$, $P > 0.5$). The probability of as few as 50 of the 177 beetles being in the clear side if they exercised no choice is less than 0.001, thus the alternative hypothesis is accepted: beetles (of either sex) avoided burrowing into the bare soil. Such a behaviour pattern, while not peculiar to female beetles laying eggs, would result in most eggs being laid in areas with plant cover, as found by Kelsey (1957). Environmental conditions in these areas are likely to be less extreme than in bare soil.

In most species of Melolonthinae "the eggs are laid singly in cells prepared by the female in small clumps of soil" (Reinhard, 1944) which are "held together by a secretion probably from the colleterial glands of the female" (Hayes, 1925). C. zealandica, however, lays its eggs "inclusters 3 to 7 in. below the surface, according to the degree of soil moisture, with 3 to 40 eggs in each cluster", each "coated with a clear sticky fluid" (Kelsey, 1951) which makes them adhere to each other and to the surrounding soil particles. A similar habit has been found in species of the tribe Sericini (Fidler, 1936a; Hoffmann, 1936). Contact of the eggs with the soil is increased as they swell during development.

Thus each stage of C. zealandica has its own place in the soil environment: the larvae in travelling cells, the pupae in their fixed cells, the beetles in crannies sheltering during the day, and the eggs packed into the soil glued together in clusters. The active stages have some choice over the regions they live in and also by their actions can modify their environment and that of succeeding inactive stages. However such interaction between the animal and its environment is limited and the basic restrictions of the soil environment remain.

PART II. THEORY

3.1 Possible approaches to the problem in the light of earlier work

Now that C. zealandica and its soil environment have been described, the way in which water in the environment affects the animal can be more clearly defined.

This is not a trivial problem; Dumbleton (1942) considered that soil moisture is "probably the most important" of the edaphic factors influencing survival of C. zealandica. Highest survival and growth rates of other scarabaeid larvae occur within a restricted range of soil moisture (Maelzer, 1961; Milne, 1963; Davidson and Roberts, 1968). Concerning soil animals in general, Collis-George (1959) stated that "the most important of the environmental factors controlling the physical behaviour of soil animals is soil-water, which largely dominates the other aspects of the environment".

An animal is under water stress when there is either more or less water in the environment than the amount favouring optimum growth. Unless change in water content is a normal part of development (as in the C. zealandica egg), animals generally tend to lose water when there is less than the optimum amount, and gain it when there is more. The first approach to the study of water relations in soil animals has been an empirical one based on observation of such loss or gain, or the associated effects on the animal's growth-rate, mortality or behaviour, at different soil water contents. Hence the conditions best suited to the animal, or conditions of minimum stress, can be determined. Studies of water relations of Scarabaeidae (as well as other soil animals) have mainly followed this approach, using a variety of ways of expressing the state of water in the soil.

The simplest measure is the percentage water content of the soil, although it is now accepted that this is only indirectly related to the moisture conditions experienced by soil animals in much the same way as the percentage concentration of solutes in water is only indirectly related to the osmotic conditions experienced by aquatic animals. Development of eggs (Kelsey, 1950; Hurpin, 1956) and movements of larvae through the soil (Granovsky, 1956; Shorey and Gyrisco, 1960)

of various scarabaeid species have been shown to be correlated with percentage water in soil.

In an attempt to reduce its dependence on soil type, soil water content has been expressed as a percentage of the amount needed to saturate the soil. Mortality of eggs and larvae, movements of larvae through the soil, water content of larvae and numbers of eggs laid by adult beetles have all been shown to be correlated with percentage of soil saturation in various scarabaeid species (Sweetman, 1931; Fidler, 1936b; Milne, 1963; Pham-Binh-Quen, 1969).

Schofield (1935) introduced the pF scale for soil moisture which is related directly to forces acting on the water in soil. Evans (1943) showed that pF could be related to the water balance of elaterid larvae in soil. In his ecological studies of a scarabaeid in soil Maelzer (1956) showed that pF was more meaningful than percentage water content. Mortality and growth of larvae, and behaviour and egg laying by adults of Scarabaeidae have since been shown to be closely correlated with soil pF (Maezler, 1960; 1961; Davidson and Roberts, 1968).

A variation of this approach has been to examine the reactions of animals in artificial environments. Mortality and weight gain or loss of Scarabaeidae of all stages have been shown to vary with relative humidity or with concentration or osmotic pressure of the bathing medium in artificial environments (Kerenski, 1930; Fidler, 1936b; Ludwig, 1936; 1946; Ludwig and Landsman, 1937; Schuch, 1938; Wigglesworth, 1945; Schäfer, 1954; Laughlin, 1957; Maezler, 1961). Unfortunately investigators using this approach have seldom related the conditions of their artificial environments to those in soil, under the assumption, usually implicit, but stated by Laughlin (1957): that "the humidity of small cavities in soil under permanent pasture must rarely if ever drop below saturation." Probably because of this misleading assumption, water content and relative humidity in soil have sometimes been treated as though they were independent (Lees, 1943a, b).

Since living organisms operate according to the same principles as inanimate systems, the rules developed to describe what happens

in physical systems can be used to predict the course of the same processes in living organisms. Thus it should be possible to predict from properties of an animal and its environment whether the animal will tend to lose or gain water, and hence, instead of deducing that the animal is under stress from observations of changes in growth-rate, survival or behaviour, to predict such changes as consequences of the known stress. This type of approach has been used, although the rationale is not usually stated explicitly, in the study of water relations of animals, particularly aquatic forms (Potts, 1954; and reviews by Brown and Stein, 1960 and Prosser and Brown, 1961). Such an approach has also been developed and used with particular success in the study of water relations of plants growing in soil (Slatyer, 1967), and could also be applied to the animals living there.

3.2 The thermodynamic approach: restrictions and advantages

The problem to be investigated was initially stated in the Introduction in terms of a rather vague concept of water stress. An animal tends to lose or gain water spontaneously when it is under water stress, and as for any spontaneous process, work must be done to arrest or reverse this movement of water. The water stress imposed on C. zealandica can therefore be considered in terms of the work the animal must do to maintain its water content. This way of reducing the problem to the interpretation of the energy relations involved in movement of water in the biological system leads naturally to the use of thermodynamic methods of analysis.

The analysis of biological systems by the thermodynamic methods leads to useful quantitative theory, subject to certain restrictions and assumptions. The first assumption which is basic in all scientific study of biological phenomena, is that living systems are subject to the same laws as inanimate systems. Next it is assumed that the particular process of interest can be considered in isolation and will move toward equilibrium unaffected by other processes occurring in the system. For many biological processes this assumption is not justified - these processes are called 'active'.

This restricts, but does not invalidate the use of thermodynamics. On the other hand the thermodynamic approach involves no assumptions about the mechanisms of the processes it deals with, which makes the analysis much simpler and wider in application. The drastic simplifications and approximations which must be made in order to analyse complex biological systems can help to isolate the essential characteristics of the problem.

The thermodynamic term 'chemical potential' (Gibbs, 1876) applied to water has several desirable properties as a measure of the state of water in the animal-environment system: it is applicable to any part of the system, it indicates the direction in which water tends to move, and it is related to the work required to reverse such movement of water. It is also measureable. The 'water potential' used in studies of water relations of plants and of soil (Slatyer, 1967; Taylor, 1968) is a practical form of the chemical potential of water. Its derivation, relations with other measureable quantities and system of units are described to make its advantages and restrictions clearer and because no standard definition is yet agreed upon. The following brief outline of thermodynamic theory leading to a definition of water potential and its properties is based on Guggehneim (1959), Babcock (1963), Spanner (1964), Katchalsky and Curran (1967) and Noy-Meir and Ginzburg (1967).

3.3 Thermodynamic theory; leading to a definition of Water Potential

Thermodynamics deals with regions of interest with definite boundaries, called systems, which have various observable properties which together define the state of the system. Properties may be extensive (dependent on the size of the system, e.g. volume) or intensive (independent of the size of the system, e.g. temperature). If the properties are not the same throughout a system it is considered as being composed of a number of homogeneous phases. In any phase there is a fixed number of independent properties which together determine all the remaining properties and hence the state of the phase.

Any change in a property of a system (or its phases) is called a process. If no process is occurring in a system isolated from its

surroundings, the system is in a state of equilibrium. A process occurring in a system only infinitesimally removed from any state of equilibrium is a reversible process; all other processes are spontaneous processes. Reversible processes are ideal processes which by proceeding infinitely slowly so that the system is always in internal equilibrium, go on indefinitely in any direction. All natural, spontaneous processes go only one way - toward a state of equilibrium, and stop when they reach this state.

If a quantity of heat q is transferred to a system from its surroundings, and work w is done by the system on its surroundings during a process, then the energy U of the system changes by an amount

$$\Delta U = q - w \qquad \ldots 1$$

ΔU is independent of the source of the heat, the form of the work, and the way in which the properties of the system change during the process.

The entropy S is an extensive property of a system, whose change dS during a reversible infinitesimal process is defined as

$$dS = q/T \qquad \ldots 2$$

During a spontaneous process

$$dS > q/T \qquad \ldots 3$$

where T is absolute temperature (with an arbitrary scale of positive values).

The work done by a system to produce an infinitesimal increase dV in volume against a pressure P is

$$w = PdV \qquad \ldots 4$$

Other work may also be done by a system during a process, such as work done in changing the composition of its phases. The composition of a phase is defined by the number of units of quantity n of each independent component i in the phase. The change in energy of the phase for each unit of quantity of component i added to the phase at equilibrium while all other properties (S, V, and quantities of all components except i - written n_j) are held constant is defined as

the chemical potential μ_i of the component i. This is written mathematically:

$$\mu_i = \left(\frac{\partial U}{\partial n_i}\right)_{S,V,n_j} \qquad \ldots 5$$

Thus the work done by a system during the change of composition of a phase is $-\sum_i \mu_i dn_i$ ...6

The total change in energy during a reversible process in an <u>open</u> phase (where any component may be added or removed), expressed in terms of the independent properties S, V, n_1, n_2, $\ldots n_i \ldots$, is therefore

$$dU = TdS - PdV + \sum_i \mu_i dn_i \qquad \ldots 7$$

In order to use a more convenient set of variables, U is transformed to a new property, G (the <u>Gibbs free energy</u>) by the relation

$$G = U - TS + PV \qquad \ldots 8$$

This may be differentiated to give

$$dG = dU - TdS - SdT + PdV + VdP \qquad \ldots 9$$

At constant temperature and pressure (the most convenient experimental conditions), the change in G during a process is

$$(\partial G)_{T,P} = dU + PdV - TdS \qquad \ldots 10$$

When change of volume is the only work done by the system on its surroundings

$$dU = q - PdV \qquad \ldots 11$$

Thus $$(\partial G)_{T,P} = q - TdS \qquad \ldots 12$$

but by definition

$$q - TdS \leqslant 0 \qquad \ldots 13$$

Thus $$(\partial G)_{T,P} \leqslant 0 \qquad \ldots 14$$

Hence G decreases in any spontaneous process and reaches a minimum value at equilibrium (where any infinitesimal process is reversible).

Substituting (7) into (9) gives an expression for the change in G during a reversible process in terms of the independent properties

T, P, n_i:

$$dG = -SdT+VdP+\sum_i \mu_i dn_i \qquad \ldots 15$$

G is an extensive property: G of a system is the sum of G of its phases. If the process consists of the movement of amounts dn of any component between any phases, at constant temperature and pressure, then

$$(\partial G)_{T,P} = \sum_k \sum_i \mu_i dn_i \leqslant 0 \qquad \ldots 16$$

where the summation is over all components and phases involved. Since any barrier between the phases which allows passage of matter will also allow passage of heat, the temperature must not only be constant but also the same in each phase - the system must be <u>isothermal</u>. If a positive quantity dn_i of a single component i is removed from phase a and added to phase b, then

$$\sum_k \sum_i \mu_i dn_i = \mu_{i(a)}(-dn_i) + \mu_{i(b)} dn_i \qquad \ldots 17$$

Thus

$$\left(\mu_{i(b)} - \mu_{i(a)}\right) dn_i \leqslant 0 \qquad \ldots 18$$

Hence if the process is spontaneous

$$\mu_{i(a)} > \mu_{i(b)} \qquad \ldots 19$$

Which means that <u>the direction of spontaneous movement is from the higher to the lower chemical potential</u>. At equilibrium, when the movement stops

$$\mu_{i(a)} = \mu_{i(b)} \qquad \ldots 20$$

The work done by the process is $\left(\mu_{i(a)} - \mu_{i(b)}\right) dn_i$ ...21

Hence if the movement is to be reversed, <u>the work required is proportional to the difference in the chemical potential between the phases</u>.

The chemical potential μ_i is one of a special class of intensive properties. From any extensive property X, the intensive <u>partial</u> property X_i is defined

$$X_i = \left(\frac{\partial X}{\partial n_i}\right)_{T,P,n_j} \qquad \ldots 22$$

Hence the chemical potential μ_i is also the partial Gibbs free energy G_i. The partial volume V_i is defined

$$V_i = \left(\frac{\partial V}{\partial n_i}\right)_{T,P,n_j} \qquad \ldots 23$$

From (15), at constant T, n_i

$$(\partial G)_{T,n_i} = VdP \qquad \ldots 24$$

or

$$\left(\frac{\partial G}{\partial P}\right)_{T,n_i} = V \qquad \ldots 25$$

Hence

$$V_i = \frac{\partial}{\partial n_i}\left(\frac{\partial G}{\partial P}\right) = \frac{\partial}{\partial P}\left(\frac{\partial G}{\partial n_i}\right)$$

$$= \left(\frac{\partial \mu_i}{\partial P}\right)_{T,n_i} \qquad \ldots 26$$

In an isothermal system whose state is completely defined by its pressure and composition, μ_i may be expressed in terms of these properties by the differential equation

$$d\mu_i = \frac{\partial \mu_i}{\partial P} dP + \sum_i \frac{\partial \mu_i}{\partial n_i} dn_i \qquad \ldots 27$$

The components of a phase are divided into water w, soluble components s, and insoluble components m. Then:

$$d\mu_i = \frac{\partial \mu_i}{\partial P} dP + \frac{\partial \mu_i}{\partial n_w} dn_w + \sum_s \frac{\partial \mu_i}{\partial n_s} dn_s + \sum_m \frac{\partial \mu_i}{\partial n_m} dn_m \qquad \ldots 28$$

Infinitesimal changes are theoretically useful but for a practical equation the integrated form relating finite quantities is used.

In an ideal gas phase

$$P_i V_i = RT \qquad \ldots 29$$

where R is the gas constant, P_i, V_i the partial pressure and volume of component i. To avoid confusion P_i is written p.

Hence $$\frac{\partial \mu_i}{\partial P}_{T,n_i} = \frac{RT}{p} \qquad \ldots 30$$

Integrating with respect to P

$$\mu_i = RT \ln(p) + C \qquad \ldots 31$$

The magnitude of the constant C is indeterminable thus only changes in μ_i can be measured. In practice a standard or reference condition is defined and all potentials measured by comparison with its potential. When the component of interest is water, pure free water with saturated vapour pressure p^o at temperature T and pressure P^o is taken as the standard and the <u>water potential</u> is defined

$$\psi = \int_{\mu_w^o}^{\mu_w} d\mu_w = \mu_w - \mu_w^o \qquad \ldots 32$$

where μ_w refers to the state with the properties P, n_w, n_s, n_m, and μ_w^o to the state where $P = P^o$, $n_s = 0$, $n_m = 0$ (i.e. pure free water).

Thus $$\psi = RT \ln p/p^o \qquad \ldots 33$$

where p/p^o is the relative water vapour pressure, which expressed as a percentage, is the relative humidity.

The expression for $d\mu_w$ (28) is an exact differential and thus its integral is independent of the path of integration. Therefore a path is chosen to give terms corresponding to the conventional measurements of the contributions of variations in pressure, soluble and insoluble components to the total water potential. The contribution due to P, the pressure or <u>hydrostatic pressure</u> component, is measured on the intact system. The <u>matric</u> contribution of the insoluble components is normally measured at standard pressure ($P=P^o$) with the soluble components also present. The <u>osmotic</u> contribution of the soluble components is conventionally measured

on an extract from the system, free of any solid matrix ($n_m=0$). Integrating equation 28 by this path:

$$\psi = \sum_s \int_{P=P^o,n_s=0,n_m=0}^{P=P^o,n_s,\ n_m=0} \left(\frac{\partial \mu_w}{\partial n_s}\right) dn_s + \sum_m \int_{P=P^o,n_s,n_m=0}^{P=P^o,n_s,n_m} \left(\frac{\partial \mu_w}{\partial n_m}\right) dn_m$$

$$+ \int_{P=P^o,n_s,n_m}^{P,\ n_s,n_m} \left(\frac{\partial \mu_w}{\partial P}\right) dP \qquad \ldots 34$$

Considering aqueous solutions as incompressible (i.e. V_w is independent of P), then

$$\int_{P=P^o,n_s,n_m}^{P,\ n_s,n_m} \left(\frac{\partial \mu_w}{\partial P}\right) dP = \int_{P^o}^{P} V_w dP = V_w(P-P^o) \qquad \ldots 35$$

where $P-P^o$ is the <u>hydrostatic pressure</u> of the phase.

The unknown matric and osmotic components of water potential in a phase (1) may be evaluated by balancing them against the pressure component of potential in a reference phase (2) which is separated from (1) by a rigid membrane permeable only to water. At equilibrium with respect to water

$$\mu_{w(1)} = \mu_{w(2)}$$

or

$$\psi_1 = \psi_2 \qquad \ldots 36$$

Now if the insoluble components are removed from phase (2) ($n_m=0$) and the pressure in phase (1) controlled at P^o, while soluble components n_s remain the same in each, then at equilibrium, movement of water produces a pressure P in phase (2) such that

$$\sum_m \int_{n_m=0}^{n_m} \left(\frac{\partial \mu_w}{\partial n_m}\right) dn_m = \int_{P=P^o}^{P} \left(\frac{\partial \mu_w}{\partial P}\right) dP = -V_w(P^o-P) \qquad \ldots 37$$

The pressure difference (P^o-P) is the <u>matric pressure</u> τ of the insoluble <u>matrix</u> in phase (1).

If insoluble components are removed from both phases and the soluble components removed from phase (2) ($n_s=0$) while the pressure in phase (1) is controlled at P^o as before, then at equilibrium, movement of water produces a pressure P in phase (2) such that

$$\sum_s \int_{n_s=0}^{n_s} \left(\frac{\partial \mu_w}{\partial n_s}\right) dn_s = \int_{P=P^o}^{P} \left(\frac{\partial \mu_w}{\partial P}\right) dP = -V_w(P^o-P) \qquad \ldots 38$$

The pressure difference (P^o-P) is the <u>osmotic pressure</u> π of the <u>solution</u> in phase (1).

Hence the total water potential may be expressed

$$\psi = V_w \Delta P - V_w \pi - V_w \tau \qquad \ldots 39$$

and can be measured from the contributions of each of these terms, or from p/p^o or other related properties. It has all the useful properties of the chemical potential: in an isothermal system water tends to move from regions of higher to regions of lower potential until equilibrium is reached when the water potential is everywhere the same, and the work involved in moving water from one region to another depends on the difference between their water potentials. Hence water potential is a convenient measure of the status of water in biological systems, subject to the restrictions and assumptions made in its derivation.

By definition, pure, free water has a water potential of zero. In biological systems, the hydrostatic pressure component of water potential is generally small compared with the osmotic and matric pressure components, thus (from equation 39) water potential is usually negative. Thus <u>lower</u> potentials corresponding to <u>drier</u> conditions have <u>larger</u> numerical values.

3.4 Units - old and new

The physical dimensions of water potential and the other terms have not been given explicitly. The way in which this is done determines the units in which these quantities are expressed.

Many units have been used for terms essentially the same as these thermodynamic quantities because workers in different fields have used the units which were familiar and meaningful to them. Unfortunately some of these units suggest false analogies and unreal concepts. This raises the problem of whether to use these familiar but obsolete units, or the theoretically best units which because of their unfamiliarity are not readily visualised or associated with the condition being measured.

The units with most theoretical justification are those of the internationally accepted Système Internationale d'Unités, or SI system. The following are not SI units for the listed thermodynamic quantities and should be regarded as obsolete:

Chemical potential:	erg/g
Pressure:	atmosphere
	bar (and subunits: millibar etc.)
	heights of columns of liquids: mmHg, cm water, etc.
	g/cm^2
	$dyne/cm^2$
Osmotic pressure:	mole/litre (molarity)
	mole/kg solvent (molality)
	^{o}C freezing point depression
	%NaCl in an isosmotic solution
	vapour pressure expressed in above pressure units
Matric pressure:	pF

Taylor's (1968) arguments against the use of atmospheres and centimeters of water or mercury as pressure units apply to all

these: "the only justification for continuing to use these units is that they are traditional and hence familiar. Such a reason seems rather weak compared to the arguments in favour of a consistent set of units that may be combined and compared readily with other units that express similar concepts." McGlashan (1968) also deprecated their use: "these units are not SI units and are not coherent with SI units. Their use is to be progressively discouraged and eventually abandoned."

In the SI system the unit of pressure (hydrostatic, osmotic or matric) is the Newton per square meter ($N\ m^{-2}$). Some of the obsolete units are now defined in terms of SI units:

$$1 \text{ atmosphere} = 101325\ N\ m^{-2}$$

$$1 \text{ bar} = 10^{5}\ N\ m^{-2}$$

$$1 \text{ mmHg} = 133.32239\ N\ m^{-2}$$

Hence it can be shown that

$$1\ cmH_2O = 98.328\ N\ m^{-2}$$

This relation can be used to convert pF units ($\log_{10}$ of negative matric pressure measured in cmH_2O) to $N\ m^{-2}$.

By expressing the 'quantity of component' in the definition of the chemical potential (page 40) in terms of quantity (in moles) or mass (in kilograms) the molar chemical potential (unit: Joules/mole) or the specific chemical potential (unit: Joules/kilogram) respectively are obtained. Under the definition given, water potential has the same units as chemical potential. Plant physiologists have generally expressed pressures in bars and used 'specific' water potential because of the numerical similarity of the units: 1 bar is numerically (but not dimensionally) equivalent to 100 $J\ kg^{-1}$ (Slatyer and Taylor, 1960). A different water potential has also been defined from equation 39 as: $\frac{\mu_w - \mu_w^{o}}{V_w}$

(Slatyer, 1967) which has the dimensions of a pressure, like its components. Relative humidity, a dimensionless percentage, can also be used as a measure of water potential (see equation 33) when it is usually expressed as a fraction and called the activity of water. In this study, water potential is expressed in J kg^{-1}.

Water potentials measured at different temperatures are not strictly comparable, but measurements not made at a standard temperature of 25°C will be corrected wherever possible to that temperature.

The water potential in the soil environment generally corresponds to humidities close to 100%. The relative humidity scale is rather misleading in this region: apparently insignificant depressions of humidity below 100%, or saturation, correspond to significant changes in the energy level of water, as measured by the water potential. Water potentials at various humidity levels are listed in Table 5. Thus the common statement that "the air in the soil is almost always saturated with water vapour" (Ghilarov, 1959; see also Ghilarov, 1968 and Kühnelt, 1963) is a misleading oversimplification.

Table 5. Humidity and water potential at 25°C.

Relative humidity, %	Water potential, J/kg
1	-633,671
10	-316,836
50	-95,377
75	-39,585
90	-14,498
95	-7,057
97.5	-3,484
99.0	-1,383
99.5	-689.4
99.9	-137.6
99.95	-68.82
99.99	-13.76
99.995	-6.880
100 exactly	0

3.5 Measurement of water potential by the Thermocouple Psychrometer

Many techniques have been developed for the measurement of water potential and its components: hydrostatic, osmotic and matric pressures. In most methods a reference phase is introduced into the system under investigation, allowed to come to equilibrium with it, and some convenient property of the reference phase (from which the property of interest can be found) is measured. Bayliss (1959), Barrs (1968), Slatyer (1967) and Boyer (1969) review various aspects of the methods used in biological systems.

The methods used in this study will be described as they arise except for the Thermocouple Psychrometer technique of water potential measurement which is described here. After some development, this technique showed considerable promise, but unfortunately gave few useful results.

The thermocouple psychrometer technique of water potential measurement depends on the relation

$$\psi = RT \ln (p/p_o) \qquad \ldots 40$$

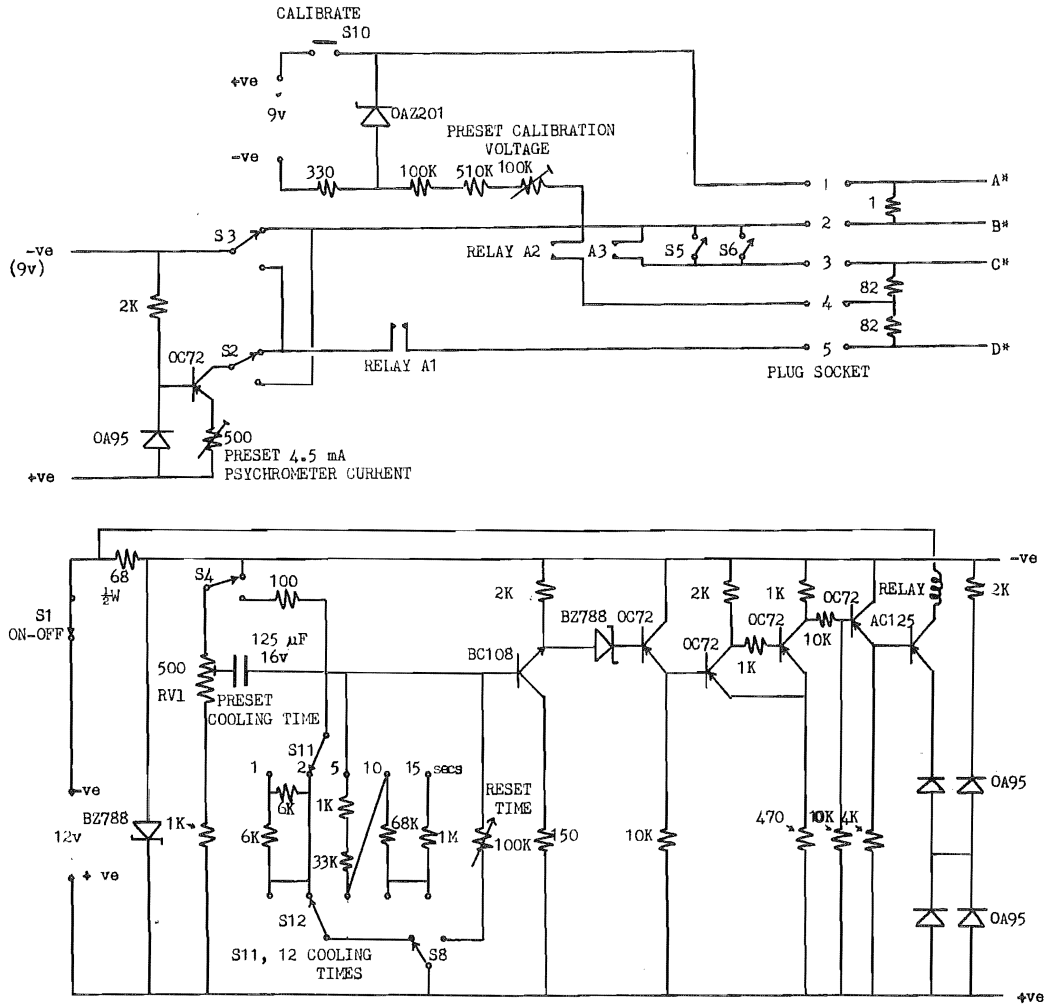
A sample is confined in a closed container with all other sources and sinks for water vapour eliminated as far as possible and its water potential found by determining p/p_o, the relative vapour pressure (relative humidity) in equilibrium with it. This is found by measuring the 'wet bulb depression' - the depression of temperature caused by the cooling effect of free evaporation of water into air of that relative humidity. The lower the humidity the more rapid the evaporation and hence the greater the wet bulb depression.

Spanner (1951) developed an ingenious technique for measuring wet bulb depression using the thermoelectric effects of metal couples. When two different metals are joined into a loop, passage of electric current around the loop heats one of the junctions between the metals and cools the other. This is the Peltier effect. Conversely, if one junction is made warmer than the other, an electric current is produced in the loop and flows in such a direction as to reduce the temperature difference between the junctions. This is the Seebeck effect, by which temperature differences can be measured with

thermocouples. Spanner's (1951) psychrometer uses a fine thermocouple with one junction exposed to the humidity to be measured and the other junction (the reference junction) connected to relatively massive copper wire so that its temperature does not change appreciably from ambient when electric current flows through it. Passing a current in the right direction cools the fine junction until the water vapour in the air begins to condense on it. The temperature of the fine junction begins to rise as soon as the cooling current is switched off, but while the water is evaporating from the junction, it remains at the wet bulb temperature. Then the difference between the temperatures of the fine and reference junctions is the wet bulb depression and may be measured from the voltage produced by the Seebeck effect. This voltage is transient because the water rapidly evaporates from the fine junction and it soon warms to ambient temperature again.

Spanner (1951) calculated that if water potential were required to an accuracy of 10 J/kg the wet bulb depression must be measured to an accuracy of about $0.001^{\circ}C$, and that temperature variations within the sample chamber must be kept to this order also. Most suitable thermocouples have outputs of less than 0.1 $mV/^{\circ}C$, so the output of the thermocouple psychrometer must be measured to an accuracy of better than 1 μV. Spanner (1951), Monteith and Owen (1958) and others used mirror galvanometers but electronic amplifiers and recording potentiometers give better results, (Box, 1965).

Most users of the thermocouple psychrometer technique have satisfied the temperature requirement given above with water baths with sophisticated control equipment. However, as Rawlins and Dalton (1967) demonstrated, the temperature need only remain constant within the required limits while the measurement is being made, provided that the humidity is in equilibrium with the sample. They dispensed with temperature control entirely and successfully relied on the thermal insulation by the soil whose water potential they were measuring to reduce temperature fluctuations. The output of the psychrometer before the cooling current is applied is subtracted from the peak output after cooling to correct for temperature differences between the sample chamber and the reference junction,



SWITCH SEQUENCE :	S1	S2/S3	S4	S5/S6	S8
FUNCTION:	ON/OFF	COOL/RESET PSYCHROMETER	START/STOP TIMING	METER PROTECTION	COOL/RESET TIMING
OFF	OPEN	FORWARD	100Ω	CLOSED	S12
READ BASE	CLOSED	FORWARD	100Ω	OPEN	S12
-	CLOSED	FORWARD	-9v	CLOSED	S12
READ OUTPUT	CLOSED	FORWARD	-9v	OPEN	S12
-	CLOSED	REVERSE	100Ω	CLOSED	100K RESET
RESET	CLOSED	REVERSE	-9v	CLOSED	100K RESET
OFF	OPEN	FORWARD	100Ω	CLOSED	S12

NOTES: S10 'CALIBRATE' PUSH BUTTON. S11/S12 GANGED TO PROVIDE FIXED COOLING TIMES: 1, 2, 5, 10, SECONDS (PRESET WITH RV1). A* CONNECTED TO D* FOR CALIBRATION, B* AND C* OUTPUT TO -VE AND +VE METER TERMINALS. PSYCHROMETER CONNECTED BETWEEN -VE METER TERMINAL AND D*.

FIG. 64. CIRCUIT DIAGRAM OF THERMOCOUPLE PSYCHROMETER CONTROL UNIT

and also for extraneous voltages in the circuit.

The output of the Spanner thermocouple psychrometer depends on the geometry of the psychrometer and the sample chamber (Rawlins, 1966; Peck, 1968, 1969) hence calibration is done empirically, using known humidities over salt solutions (Lang, 1967). A standard current flowing for a standard time must be used for the Peltier cooling, and precautions must be taken against extraneous voltages arising in the circuit, particularly in switch contacts, since voltages developed at such junctions may be greater than the voltage to be measured.

Water potential measurements were made using thermocouple psychrometers (built by Mr E. Campbell, Logan, Utah, U.S.A.) made of Chromel-Constantan couples (welded from 0.001 inch thick wire) connected to copper wire leads. The cooling current and timing, and switching of the output were controlled electronically by a circuit designed and built by Mr N. Galbreath. This control unit allowed the Peltier current to be reversed to heat the psychrometer fine junction after use (so as to evaporate any water remaining on it) and provided a known voltage (10 μV) for calibrating the recording system. A circuit diagram of this control unit is given in fig. 64.

The psychrometer output was amplified and measured with either a Kipp and Zonen 'Micrograph BD 2' (full scale deflection on the lowest range 50 μV) or a Hewlett-Packard '419-A Microvoltmeter' (full scale deflection on the lowest range 3 μV). The accuracy of the measurements was limited by the inherent electrical noise of the instruments: 0.15 μV in the microvoltmeter, and a similar amount, concealed by the 'dead zone' in the Micrograph.

Glass sample chambers were originally used with the thermocouple psychrometer (Spanner, 1951; Monteith and Owen, 1958; Korven and Taylor, 1959) but equilibrium is reached more rapidly in metal or Teflon chambers (Lambert and Schilfgaarde, 1965). Equilibration in glass chambers is slow because water vapour is adsorbed on glass surfaces forming layers up to 500 Å (50 nm) thick (Garbatski and Folman, 1956), and is absorbed into the glass as well (Haller, 1960). Boyer (1967) showed that equilibration was accelerated even in a

brass chamber when "the inner surface of the chamber was made hydrophobic with a coating of vaseline", so a similar approach was used to try to improve the performance of glass chambers, which were far more convenient than metal ones. The inner surfaces of small (4x1 cm) glass bottles were treated with dimethyldichlorosilane, which makes glass surfaces hydrophobic (Holland, 1964), and the bottles sealed with carefully cleaned polythene caps pierced to fit tightly around the base of the psychrometer assembly. The complete sample chamber was sealed by smearing vaseline over the joins, and then suspended by the insulated psychrometer lead wires in a water bath. No further temperature control was needed; temperature changes in the bath were slow. When a solution of known water potential was placed in the bottom of one of these chambers, equilibrium humidity (indicated by a constant psychrometer reading) was reached in about six hours. Without the dimethyldichlorosilane treatment, the psychrometer output was still drifting after several days.

3.6 Application of the theory; some restrictions

To return for a moment to C. zealandica, struggling to survive and grow in its inconstant environment: the difference in water potential between the animal and its environment indicates the direction in which water will flow and the force which the animal must overcome to maintain a constant water content. Water moves inexorably down gradients of potential and, short of shifting to a better place, any effort the animal makes to stem the flow requires continual work, like bailing out a leaking boat. Thermodynamic theory gives only the minimum work needed for an ideal, reversible process; in practice some work is wasted in bailing because the pump is not perfectly efficient.

Active transport processes, which do not proceed to the equilibrium position predicted by thermodynamic theory, are characteristic of living, metabolising organisms. Rosenberg (1954) defined active transport as "the transport of substances across one or more cell membranes which is influenced not only by the force

responsible for passive diffusion but also by other forces which are maintained and regulated by the metabolism of the cell". Usually, when these 'other forces' can be described, the process is no longer called active and "the term "active transport" is, therefore, an expression of our inability to describe the process fully in quantitative terms" (Curran and Schultz, 1968). Rosenberg (1954) considered that "the demonstration of transport from a lower to a higher potential...is the only certain criterion of active transport". This criterion is very useful in experimental studies although it excludes some processes which are active under the definition (when transport is 'downhill' - from higher to lower potential) and includes others which are not usually considered active (such as some processes in non-living systems).

Active processes are a continual drain on the energy resources of an organism, hence the notion of stress on an organism working to maintain its steady state. The effect of such processes on the movement of water means that gradients of water potential which indicate the direction of passive movement will not predict the actual direction of movement of water in the system. However, the difference between the water potentials of the animal and its environment remains a measure of the stress on the animal.

Another assumption made in the theoretical treatment which is often violated in biological systems is that the system is isothermal. Differences between water potentials are physically undefined and can not be given absolute numerical values when the potentials refer to phases at different temperatures (Spanner, 1964). The extent of the uncertainty depends on the temperature difference. All respiring organisms produce heat and are therefore unlikely to be isothermal with their surroundings.

3.7 An extension to the theory; Irreversible Thermodynamics

Non-equilibrium situations such as those due to active processes or temperature gradients can be described by the 'thermodynamics of irreversible processes'. In non-equilibrium situations processes are continually occurring and the basic equations of irreversible

thermodynamics express the rates of these processes. These phenomenological equations, like all thermodynamic equations, merely describe observable changes without implying any particular mechanism for them. They take the form:

$$J_1 = L_{11}X_1 + L_{12}X_2 + L_{13}X_3 + \cdots$$

or

$$J_i = \sum_j L_{ij}X_j \qquad \ldots 41$$

where J_i is the flux (rate of flow) of component i, X_i is the corresponding force or gradient promoting movement of i, and L_{ij} is the coefficient allowing for the influence of the gradient of j on the flux of i.

The fundamental theorem of irreversible thermodynamics is that provided J_i and X_i are measured in such a way that the rate of entropy production dS/dt of the irreversible process can be expressed as $\sum_i J_i X_i$, then $L_{ij} = L_{ji}$ (Onsager, 1931a,b).

3.7.1 Thermo-osmosis

When there is a temperature gradient within a system there will be a flow of heat. The phenomenon of a flow of water in response to a flow of heat between phases is called thermo-osmosis. Cary (1963) showed experimentally that Onsager's relations were valid and that the equations of irreversible thermodynamics adequately described the process of thermo-osmosis across an air space between liquid phases differing in temperature by up to 5°C. The rates of heat and mass flow by thermo-osmosis may be expressed in phenomenological form (Spanner, 1954, 1964):

rate of mass flow

$$J_m = L_{mm}\frac{V_w \Delta P}{T} + L_{mQ}\frac{\Delta T}{T^2} \qquad \ldots 42$$

rate of heat flow

$$J_Q = L_{Qm}\frac{V_w \Delta P}{T} + L_{QQ}\frac{\Delta T}{T^2} \qquad \ldots 43$$

under isothermal conditions ($\Delta T = 0$)

$$J_m = L_{mm}\frac{V_w \Delta P}{T} \qquad \ldots 44$$

$$J_Q = L_{Qm} \frac{V_w \Delta P}{T} \qquad \ldots 45$$

Then the amount of heat associated with the flow of unit quantity of water across the membrane between the phases is defined as the heat of transfer Q^*.

$$Q^* = \frac{J_Q}{J_m} \qquad \ldots 46$$

When ΔP and ΔT reach values such that the flow of water ceases, then

$$\frac{\Delta P}{\Delta T} = - \frac{Q^*}{V_w T} \qquad \ldots 47$$

This is a steady state, requiring a continuous flow of heat to maintain the water balance. Thermo-osmosis due to the heat produced by a respiring animal is thus, in a sense, an active process. It has been demonstrated in a biological system by Vetß (1966).

The magnitude of the thermo-osmotic effect depends on the heat of transfer Q^*. In general this can be found from the temperature coefficient (Q_{10}) of permeability of the membrane. Permeability is determined from the rate of flow of water f_o across a membrane under a standard pressure difference P_o (or other equivalent water potential difference) at constant temperature T_o. Applying the Arrhenius equation (Glasstone, Laidler and Eyring, 1941) to this rate: $f_o = K \exp\left(- \frac{E}{RT_o}\right)$ ...48

where K is a constant, E the observed activation energy of the process, and R the gas constant.

If the temperature is raised 10^o to a new temperature T_{10} the pressure difference becomes P_{10}, where

$$P_{10} = P_0 \frac{T_{10}}{T_0} \qquad \ldots 49$$

(assuming no change in volume).

Corresponding to the new rate of flow f_{10} is a new permeability

f_{10}/P_{10}. The ratio of the two permeabilities is the temperature coefficient Q_{10}:

$$Q_{10} = \frac{f_{10} T_0}{f_0 T_{10}} \qquad \ldots 50$$

Hence from equation 48

$$\frac{f_{10}}{f_0} = \frac{Q_{10} T_{10}}{T_0} = \exp\left(- \frac{E_{10}}{RT_{10}} + \frac{E_0}{RT_0} \right) \qquad \ldots 51$$

or

$$\ln\left(\frac{Q_{10} T_{10}}{T_0}\right) = \frac{E_0}{RT_0} - \frac{E_{10}}{RT_{10}} \qquad \ldots 52$$

But

$$E = RT - \Delta H \qquad \ldots 53$$

where ΔH is the heat of activation (Glasstone et al., 1941) which here is the heat of transport Q^*, thus

$$\ln\left(\frac{Q_{10} T_{10}}{T_0}\right) = \frac{RT_0 - Q^*}{RT_0} - \frac{RT_{10} - Q^*}{RT_{10}} \qquad \ldots 54$$

or, since $T_{10} - T_0 = 10$

$$\ln\left(\frac{Q_{10} T_{10}}{T_0}\right) = \frac{10Q^*}{RT_0 T_{10}} \qquad \ldots 55$$

An equivalent derivation of this equation was given by Spanner (1954). He made the further approximation that at a temperature T near room temperature, equation 55 becomes approximately:

$$\ln(1.034\ Q_{10}) = \frac{10Q^*}{RT^2} \qquad \ldots 56$$

PART III. EXPERIMENTAL

The next step in the analysis is to consider the differences in water potential of C. zealandica and its soil environment which promote movement of water from one to the other. The boundary between these two phases of the system is at the surface of the integument, including its invaginations into the alimentary canal and tracheal tubes.

4.1 The water potential of C. zealandica

All the stages of C. zealandica contain a large amount of water: 82.7% of total weight in a sample of 183 second instar larvae and 75.5% in a sample of 40 third instar larvae. Much of this water is in the body fluid or haemolymph which surrounds the internal organs and is separated from the outside environment only by the thin integument. Water potential can be conveniently measured from the haemolymph. However removal of haemolymph from the animal eliminates the hydrostatic pressure component of its water potential, so this must be estimated separately. There is no simple method for measuring hydrostatic pressure in small organisms, partly because the pressure is normally low. For the same reason it may be expected to contribute little to the total water potential. Higher pressures may occur in the egg as it absorbs water and expands.

The hydrostatic pressure within the egg of C. zealandica was estimated from how much the egg flattened under a known load. The method is derived from that of Cole (1932) and Browning (1967) in which the pressure is calculated from the force exerted by the load and the area it acts upon. A glass slide was lightly smeared with oil and rested at one end on the egg. The oil held the egg by surface tension while the slide was lifted and the apparent area of contact with the egg (made more clearly visible by the oil meniscus) measured with a calibrated microscope. The difference between this area and that measured with the weight of the slide resting on the egg gave the area supporting the load. The extra load on the egg would increase its pressure. This increase ranged from 15% to 180%

Table 6. Water potential of haemolymph of C. zealandica.

Stage	Number	Water potential in J/kg	
		mean	standard deviation
egg	9	-922	237
first instar larva	8	-719	80
second instar larva	4	-816	62
(newly moulted third instar larvae: -605 and -615 J/kg)			
normal third instar larva	13	-867	75
prepupal third instar larva	5	-982	175
pupa	6	-893	27
(teneral adults: -960, -960, -961, -1632 J/kg)			
mature adult	10	-1007	123

Table 7. Water potential of scarabaeid haemolymph.

Species	Potential in J/kg	Author
Eggs:		
Phyllopertha horticola	-730 to -1390	Laughlin (1957)
Larvae:		
Melolontha vulgaris	-925 to -1050	Rouschal (1940)
Melolontha sp.	-1050 to -1210	Vialli (1925)
Oryctes nasicornis	-1000 to -1015	Vialli (1925)
Prepupal larvae:		
Popillia japonica	-1270 to -1350	Ludwig (1951)
Adults:		
Melolontha vulgaris	-1160	Rouschal (1940)

in Cole's (1932) measurements. A swollen C. zealandica egg supported a load of 3.268 gm on an area of 7.9×10^{-3} cm^2, so allowing for an increase of 100% under the load, the hydrostatic pressure of the egg contents was approximately 2.03×10^4 N m^{-2} (0.2 atmosphere).

The freezing point of an aqueous solution is a function of its water potential. The osmotic and matric components of water potential of C. zealandica haemolymph (the hydrostatic pressure component is eliminated when the haemolymph is removed) were measured by the freezing point depression technique using the method of Ramsay and Brown (1955). This method is fairly accurate and requires only small samples of haemolymph, but is slow. Water potentials of 61 C. zealandica, including all the stages of the life cycle, were calculated from measurements of the freezing points of their haemolymph. The mean and standard deviation of the potentials of each stage, and individual potentials of the few newly moulted third instar larvae and teneral adults measured, are given in Table 6. The potentials do not vary much except in animals near ecdysis (newly moulted third instar larvae, prepupal larvae, and teneral adults), when the physiological upheaval apparently affects the haemolymph. The egg and adult are the most variable stages. The egg more than doubles its water content during development so variation in its water potential is to be expected, but there was no decreasing trend as Laughlin (1957) found during the development of Phyllopertha horticola eggs. The variation in water potential in the adult may be a result of rapid desiccation when it moves out of the soil into the open air, where it is poorly protected by a cuticle almost as permeable as those of the other stages which always remain in the soil. The water potential of C. zealandica is similar to that of other Scarabaeidae at the same stage, as Table 7 shows.

Water potentials of C. zealandica larvae were also found by confining larvae in a closed space and measuring the humidity of the air with a thermocouple psychrometer when it reached a steady state. Measurements from live third instar larvae were erratic and higher by as much as 530 J/kg than the mean of the freezing point depression

measurements.

This discrepancy may be produced by thermo-osmosis. In the experimental conditions, and also in the natural soil environment, the 'membrane' between the liquid water in C. zealandica and in its surroundings includes the air space around the animal as well as its integument. Temperature gradients will produce thermo-osmotic effects across both of these. The temperature gradient and heat of transport, which determine the magnitude of the effect are both difficult to measure for the cuticle, but much simpler for the air space. The heat of transport in this case is the heat abstracted from one phase to evaporate the water and added to the other phase when the water condenses there: the latent heat of evaporation of water (Spanner, 1954). Thus the pressure difference (or equivalent water potential difference) across the air space at steady state can be calculated using equation 47 as 79.6×10^5 N m^{-2} (over 80 atmospheres) for a $1^{\circ}C$ temperature difference, with the higher pressure on the cooler side.

The temperature gradient in the air around C. zealandica larvae produced by heat from their respiration was estimated from thermocouple measurements. Each larva was placed in a closed bottle with a thermocouple psychrometer mounted through the lid. When the psychrometer reading became steady, indicating that evaporation from the larva had reached steady state, the psychrometer was pushed down into the bottle until its fine thermocouple junction (about 0.1 mm in diameter) touched the cuticle of the larva, and then withdrawn. The change in the output of the thermocouple, recorded on a chart recorder, was then a measure of the temperature difference between the cuticle surface and the air some distance above it. Four successive measurements of the difference between air and cuticle surface temperatures on an active third instar larva were 0.27, 0.21, 0.22 and $0.19^{\circ}C$ (mean $0.22^{\circ}C$). A temperature difference of this magnitude leads to a steady state pressure difference of 17.5×10^5 N m^{-2} by thermo-osmosis. For this larva, thermo-osmosis would therefore make the environment effectively 1750 J/kg drier than its actual water potential.

The magnitude of the thermo-osmotic effect across the cuticle is hard to estimate. Permeability of insect cuticle increases with temperature (Wigglesworth, 1945) i.e. its Q_{10} is positive, so thermo-osmosis across the cuticle probably adds to the pressure difference given. Hence the measured water potential of this larva at steady state will be higher by up to 1750 J/kg or more than its actual water potential. Thus thermo-osmosis could well account for the discrepancies of the order of 500 J/kg in the measurements of water potential of active larvae.

To stop all active processes including thermo-osmosis, larvae were killed by freezing. This method was used in order to kill the larvae without changing their composition or structure, although it eliminates their internal hydrostatic pressure. Freezing is used for the same reasons in measurement of water potential of plants (Barrs, 1968). Thermocouple psychrometer measurements of the water potential of the dead larvae were more consistent: the potentials of six larvae were -600, -616, -743, -859, -961 and -1062 J/kg. The mean of these measurements (-807 J/kg) is similar to that measured from haemolymph by the freezing point depression method (-867 J/kg), which suggests that both methods measure the water potential of the whole animal, and gives support to the hypothesis that the water potentials of living larvae were altered by thermo-osmosis or other active processes.

4.2 The water potential of the soil environment

Water in soil is often expressed as a mass fraction (i.e. percentage soil moisture) since this is so simple to determine. Unfortunately the relation between τ, the matric component of the water potential, and n_w/n_i, the mass fraction of water in soil is non-linear, varies considerably with the structure of the solid matrix (and hence varies between different soils, or undisturbed and sieved soil of the same type) and shows considerable hysteresis - the form of the relation is not constant, but depends on the previous history of the variables. This relation is called the water characteristic curve of the soil.

The other components make up little of the water potential

in soil. In unsaturated soil the hydrostatic pressure is zero. The osmotic pressure varies with the water content but is low in most soils. The osmotic pressure of soil solution filtered from saturated West Melton soil was determined from its electrical conductivity. The conductivity of the solution at 17°C was 0.59 millimhos per centimeter ($5.9 \times 10^{-6} \Omega^{-1} m^{-1}$ in SI units) which converted by the relation given by Richards (1954) corresponds to an osmotic pressure of 2.04×10^{4} N m^{-2} (0.202 atmosphere). The osmotic pressure of the soil solution rises in approximately inverse proportion to percentage soil moisture up to the limits of solubility of the salts in solution.

The difference between the water potentials of C. zealandica and its soil environment is a measure of the water stress on the animal, but will not predict the actual direction of movement of water because of thermo-osmosis and other active processes. Therefore accurate measurements of soil water potential are of limited use and for most purposes the simple method of estimating soil water potential from percentage water content using the water characteristic curve is sufficiently accurate, despite all its limitations.

The water characteristic curve for the soil inhabited by C. zealandica at West Melton was determined by a variety of methods each suitable for a different range of matric pressure: 0-1.5×10^{3} N m^{-2}.

A method similar to that of Meats (1967) was used. Three 15 cm lengths of 2 cm internal diameter Neoprene tubing were filled with soil that was broken up as little as possible, and placed vertically in a jar full of water until the soil was completely flooded. Then water was siphoned out, leaving only the bottom centimeter of the tubes of soil standing in water. The jar was sealed and left for ten weeks, then the Neoprene tubes were cut into 1 cm lengths and the water content of the soil in each segment determined by the standard procedure (measuring loss in weight after 24 hours in an oven at 105°C). Assuming the water in the soil columns to be at equilibrium, the matric pressure in each segment depends on its

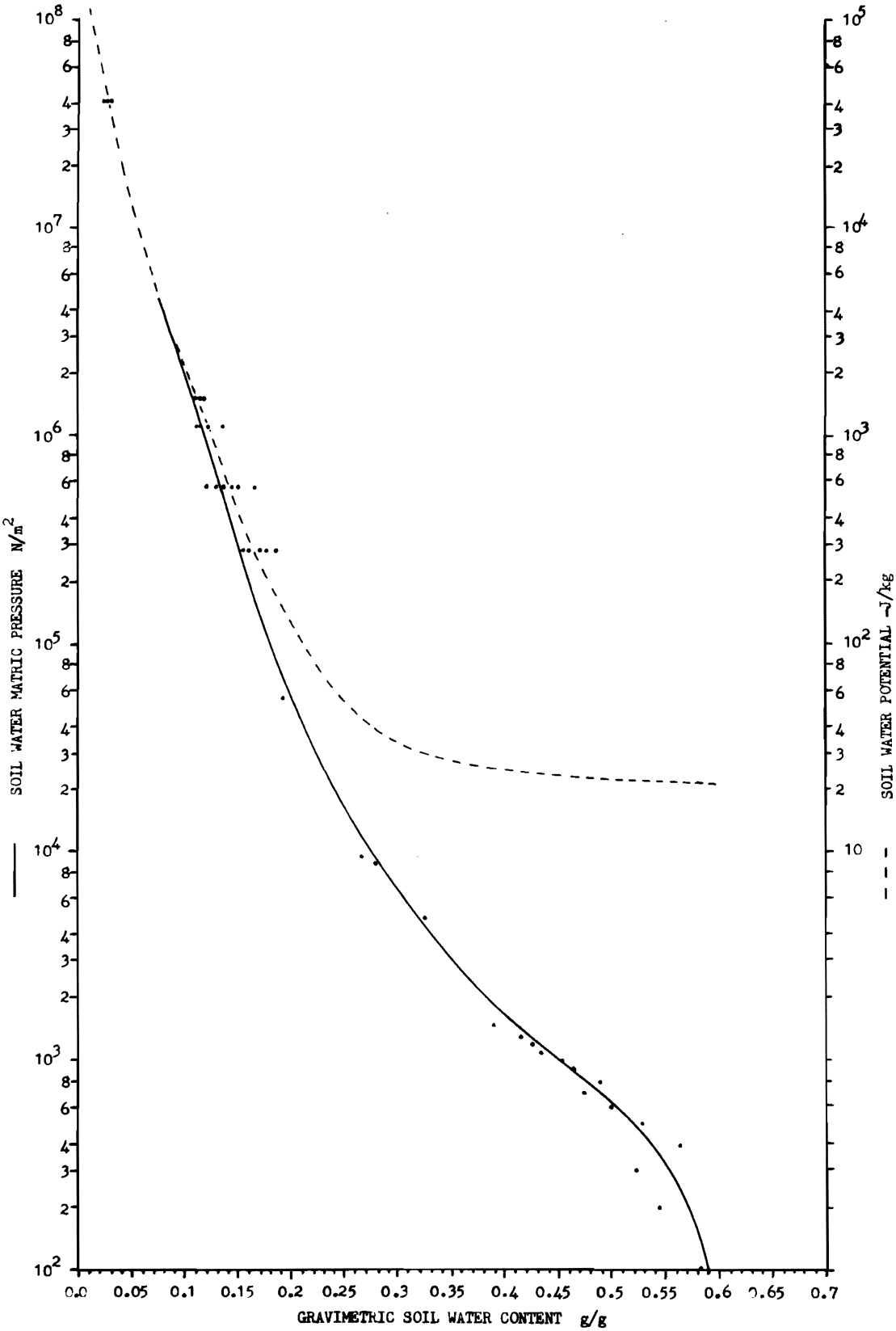


FIG. 65. WATER CHARACTERISTIC CURVE OF WEST MELTON SOIL.

height above the free water surface.

The following are standard methods, all described by Croney, Coleman and Bridge (1952):

1.5-55 x 10^3 N m^{-2}. The Suction Plate method.

Millipore filters (0.47 μm pore size) were used for the porous plate. The suction was controlled by varying the height of the water column and using a suction pump and mercury manometer only for the highest suction. At equilibrium the matric pressure in the soil depends on the height of the water column (or its equivalent when the mercury manometer was used) above the free water surface.

200-1500 x 10^3 N m^{-2}. The Pressure Membrane method.

The apparatus used was a 'Pressure membrane extractor' (Soilmoisture Equipment Co). Discs of undisturbed and sieved soil about 4 cm in diameter and 1 cm thick were used, and subsamples were removed for water content determination after equilibration at each successively higher pressure. At equilibrium, the matric pressure in the soil depends on the pressure in the apparatus. Measurements from undisturbed and sieved soil fell within the same range.

Over 1500 x 10^3 N m^{-2}. The Vacuum desiccator method.

Soil samples were suspended over saturated sodium chloride solution (equilibrium humidity 75.5% at 20°C) in a flask evacuated by water pump to a pressure a little above the vapour pressure of water, and left for two weeks to come to equilibrium. Then the total water potential (not just the matric pressure) in the soil depends on the relative humidity in the flask.

The water characteristic curve determined by these methods is given in fig. 65. Different samples of soil both within and between methods gave slightly different curves, hence the sections of the curve determined by different methods do not join exactly. This is a reflection of the variability of the soil as well as experimental error. Also in fig. 65 is the curve relating soil water content and total water potential, obtained by adding osmotic and matric components, assuming an inverse relationship between osmotic pressure of soil solution and soil water content. This curve will be used to convert percentage soil moisture measurements to water potentials.

Since the water content of soil in the field at any time is highly variable (Taylor, 1955), considerable replication is required to obtain reliable estimates, representative of the conditions faced by the animals living in the soil. Thus taking samples of soil for water content or water potential determination is, as Holmes (1961) expressed it, "laborious and time consuming, and moisture measurements at a 'point' are obtained. In addition repeated sampling slowly removes the plot area into the laboratory." Indirect determination, from climatological data, of the variation of soil water content during the year is adequate to demonstrate the pattern of water stress faced by C. zealandica.

Several methods have been developed by which the amount of water in the soil can be calculated as the difference between the amount added by rain, and the amount lost by evaporation, transpiration from plants, surface runoff, and percolation into deeper layers. Baier (1968) reviewed some of these methods and demonstrated that the method of Thornthwaite (1948) as modified by Thornthwaite and Hare (1955) gave the best approximation to the annual trend of soil water content under short grass. This method uses an empirical estimate (from mean daily air temperature) of "potential evapotranspiration", or "the evapotranspiration that would occur from a vegetation covered surface if soil moisture conditions were adequate for unrestricted transpiration" (Thornthwaite, 1948). This estimate depends on the mutual correlation of both temperature and evaporation with radiation, and is sufficiently reliable only over long periods (Pelton, King and Tanner, 1960). Thornthwaite's method has given reliable estimates of seasonal changes in soil water content in pastures in many areas (Mather, 1954; Smith, 1959) including Winchmore, South Canterbury, 40 miles to the south of West Melton (Rickard, 1957, 1960; Fitzgerald and Rickard, 1960).

Soil water content at West Melton during the years 1968 and 1969 was calculated by Thornthwaite's method using weekly rainfall (in inches) and mean air temperature (in ^{o}F) taken from the Meteorological records for Christchurch Airport, which is 8.5 miles away from West Melton. Only the top 23 cm layer of soil was

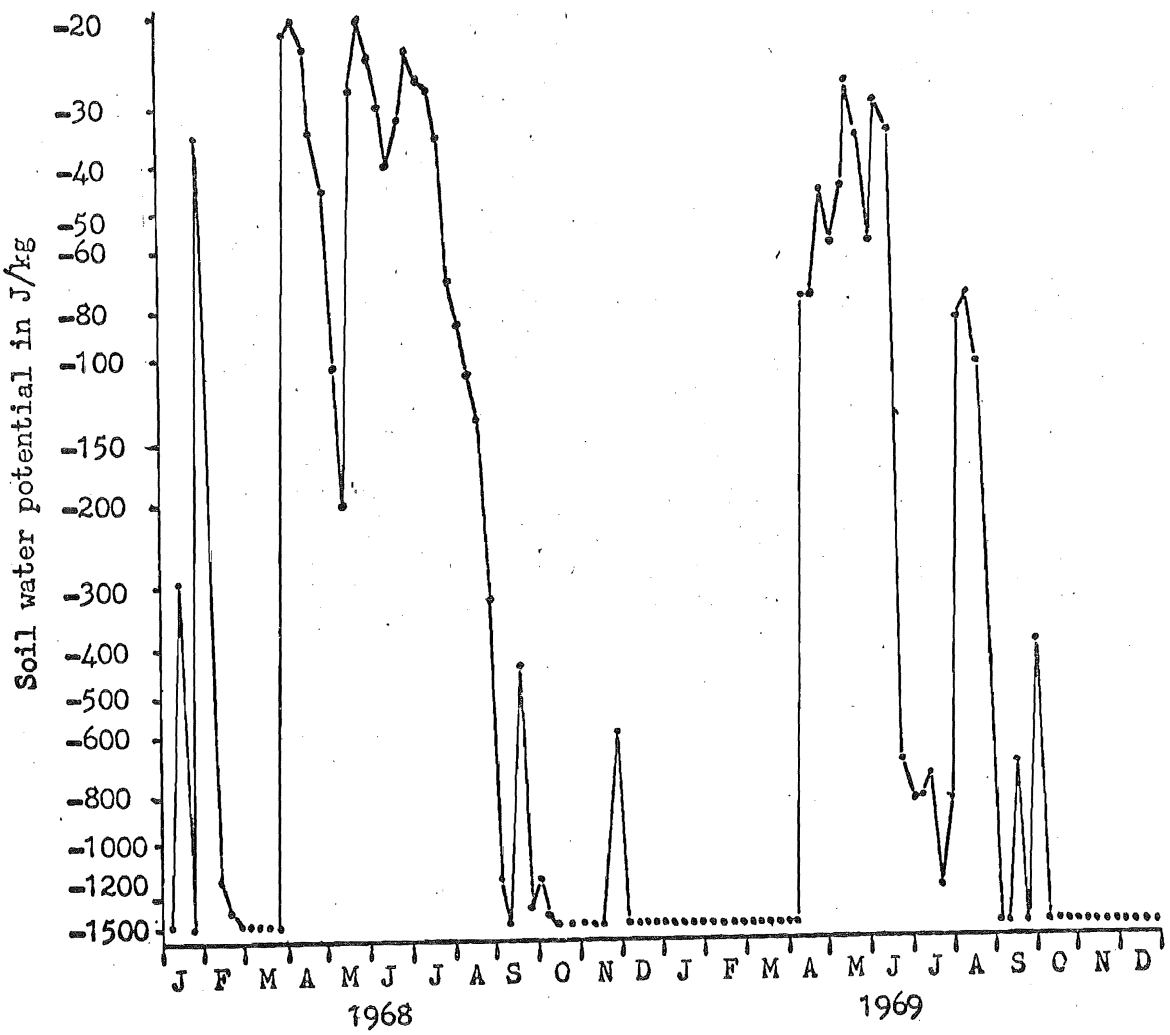


Fig. 66. Estimated soil water potential at West Melton during 1968 and 1969.

included; no C. zealandica and very few plant roots were found in the sandy layer below this. The dry bulk density of the top layer (found by weighing a sample taken with a soil corer after drying for 24 hours at 105°C) was 1.07 gm/cm^3. The percentage water content of the soil at 'field capacity' (matric pressure 0.2 x 10^5 N/m^2) and at 'wilting point' (matric pressure 15 x 10^5 N/m^2), taken from the water characteristic curve in fig. 65 are 25.0% and 11.5% respectively. Hence the 'water holding capacity' of the top 23 cm layer of soil is calculated as 3.3 cm. Weekly estimates of percentage soil water content were made from these parameters by Thornthwaite's (1948) method using the tables of potential evapotranspiration given by Thornthwaite and Mather (1957), and converted to soil water potential using the relation in fig. 65. A graph of the estimated soil water potential at West Melton during 1968 and 1969 is given in fig. 66.

4.3 The water stress on C. zealandica, and how it compares with that on other animals.

The results presented show that the water potential of C. zealandica varies little within any stage, or from stage to stage during the year even though the water potential of its soil environment fluctuates considerably and has a seasonal cycle. At various times the potential of the animal ranges from about 800 J/kg lower to more than 800 J/kg higher than that of its environment, which shows the extent to which C. zealandica may be under water stress.

The soil at West Melton is generally dry when C. zealandica eggs or first instar larvae are present, but it fluctuates between wet and dry at the time of the second instar larvae. The soil is generally wet for the third instar larvae but again fluctuates between wet and dry as they pupate, and is mainly dry when the adult is active. However weather conditions are so variable that any stage may encounter extreme wet or dry conditions. A shower of rain during a dry spell can change the soil water potential and hence the water stress on the animal from one extreme to the other.

Other insects live under much greater water stress, but few withstand reversal of the direction of stress. For instance, Tenebrio molitor neither gains nor loses water at 88% relative humidity (-17,500 J/kg water potential) but in humidities near saturation its water balance is upset and it gains water uncontrollably (Mellanby, 1932).

The water regime in soil may be compared with that in some aquatic environments. Soil animals are protected against the rapid diffusion of water suffered by aquatic animals by the air space around them because diffusion through air is slower than through water "by a factor of about 10,000" (Meidner and Mansfield, 1968). In experiments in which soil animals are placed in solutions this protection is eliminated. Ramsay (1949) concluded from such experiments that "the osmotic relations of the earthworm are such as are characteristic of freshwater animals generally". However, this does not mean that with respect to water, soil is like a freshwater environment. Fresh water has a water potential near zero. In coastal sea water the normal range of water potential is from -2700 to -2300 J/kg (Robertson, 1960); sea water with a water potential of -1500 J/kg (equivalent to 'wilting point' in soil) has a salinity of about 60% of normal sea water (Barnes, 1954, Robinson, 1954). Salinity of the water in an estuary may reach this level or more at high tide and fall to fresh water levels at low tide (Alexander, Southgate and Bassingdale, 1932). Thus the water potential regime in soil is comparable to that in an estuary, although the water stress in soil is tempered by the air space around the animal.

4.4 Responses of animals to water stress.

The remainder of this study deals with some of the ways in which C. zealandica reacts to the water stress it encounters. Large populations survive in pastures throughout the year, so this stress is evidently not normally fatal.

There are only a few possible responses an animal can use to successfully counteract the adverse effects of water stress. The

Table 8. Changes in weight of C. zealandica larvae surviving one week in soil of known water content.

Soil water		Final weight of larvae as percentage of initial weight												
%;	potential J/kg	1st instar			2nd instar			3rd instar						
2	-100,000	-	-	-	46	-	-							
3	-35,000	52	46	-	73	54	71							
4	-20,000	68	61	-	73	54	71	80	82	86	76	-		
5	-12,000	64	68	80	70	79	73							
6	-8,500	68	56	-	79	77	64	84	85	95	-	-		
7	-6,000	77	-	-	81	-	-	96	91	95	-	-		
8	-4,200	62	-	-	96	92	-	94	97	96	94	-		
9	-3,000							92	97	85	97	-		
10	-2,400	89	85	-	91	83	-	99	99	96	-	-		
12	-1,300							105	101	94	98	-		
16	-320							93	88	-	-	-		
20	-110							106	101	100	98	-		
24	-60							100	98	97	95	-		
28	-35							105	100	98	94	87		

Water potentials estimated to two significant figures. Dashes indicate larvae which died during the experiment.

animal may tolerate the change in water content resulting from the stress and allow its water potential to change with that of the environment. It may regulate change in water content, either passively by insulating itself to slow water gain or loss, or actively, compensating for gain or loss by pumping water up the potential gradient, and maintain its potential more or less independent of the environment's. Or, it may avoid the stress by moving to a less hazardous place.

Such responses help the animal to survive, not only through the stage making the response, but through succeeding stages of the life cycle as well. Survival of stages with fewer defences (such as immobile stages, which can not avoid stress) may depend on protective responses made for them by the preceding stage. Each stage of C. zealandica uses a combination of responses to deal with water stress.

4.5 Responses of C. zealandica larvae to water stress

4.5.1 Weight changes of larvae under water stress in soil

In a series of experiments, samples of the three larval instars of C. zealandica were weighed before and after one week in sieved West Melton soil with a range of water contents. There were no growing plant roots in the soil so that apart from some weight changes from ingestion of soil and defecation, gain or loss of water should produce the main trend of weight change. The changes in weight of the surviving larvae are listed in Table 8.

Since the water potentials of the larval instars (measured from their haemolymph) were mainly between -700 and -1000 J/kg, gain of water is expected in soil water potentials above -700 J/kg, and loss in potentials below -1000 J/kg, if there is no controlling mechanism. In the third instar larvae, erratic weight changes obscure any such trend except in the driest soils. Weight changes from water gain or loss in soils with potentials from -35 to -6000 J/kg must be small compared with those from other causes. Only in soil drier than about -6000 J/kg did larvae consistently lose more than 10% of their initial weight. First and second instar larvae

Table 9. Changes in weight of C. zealandica larvae floating on sucrose solutions.

Second instar larvae:

Time in hours from start:		0	17	24	40	48	64	72	81
Sucrose solution		Final weight of larva as percentage of initial weight							
concentration mol/kg	water potential J/kg								
0.05	-120	100	124	124	122	132	141		
0.2	-480	100	107	113	126	127	133		
0.4	-960	100	96	93	97	95	99	98	94
0.6	-1440	100	95	86	82	78	74	72	64
0.8	-1920	100	86	75	68	65	62	61	57
1.0	-2400	100	85	70	60	56	50		
2.0	-4800	100	80	62					

Third instar larvae:

0.0	0	100	117	118					
0.05	-120	100	108	109					
0.1	-240	100	110	110	116	117			
0.2	-480	100	108	107	112				
0.3	-720	100	104	103					
0.4	-960	100	108	101	109	108			
0.5	-1200	100	98	96	94	94	92	94	89
0.6	-1440	100	103	96	92				
0.7	-1680	100	95	83	78	76	72	69	67
0.8	-1920	100	94	87	84				
0.9	-2160	100	97	89	84	86			
1.0	-2400	100	95	88					
1.5	-3600	100	101	88	79	79	72	70	62

Table 10. Changes in weight of C. zealandica larvae floating on glycerol solutions.

Concentration mol/kg	Water potential J/kg	Number gaining weight;	losing
0.30	-720	9	1
0.325	-780	8	1
0.35	-840	4	2
0.375	-900	8	2
0.40	-960	4	5
0.425	-1020	1	5
0.45	-1080	4	5
0.475	-1140	2	8
0.50	-1200	2	7

lost weight more rapidly than third instar larvae but still survived considerable water stress. Even in a water potential of -100,000 J/kg one second instar larva still survived, while losing more than half its initial weight.

4.5.2 Weight changes of larvae under water stress floating on artificial solutions

Since water potential can be controlled more simply and accurately in solutions than in soil, weight changes of larvae were measured in a further series of experiments with the larvae floating on solutions of known potential. Each second or third instar larva floated on a solution in a separate bottle and was weighed at daily or shorter intervals. Flaccid larvae which did not respond when their antennae were touched were considered dead and discarded. To reduce permeation of the solutes into the larvae, non-ionic compounds of fairly high molecular weight (sucrose or glycerol) were used in solutions of known concentration. The temperature during the experiment was controlled at 20°C. The weight changes of 7 second instar and 13 third instar larvae and the calculated water potentials of the solutions are given in Table 9.

The results of these experiments are different from those of the previous series which used soil. Here, despite the possibility of weight changes from drinking or defecation, there is a clear trend of loss of weight on solutions with potentials of -1200 J/kg or less bars and gain at -720 J/kg or more. These weight changes were far more rapid than those in soil.

In a further series a narrower range of water potentials was used and the tendency of larvae to gain or lose weight was recorded. Ten third instar larvae were used at each potential, but some whose weight oscillated about their initial value were discarded. The concentration and water potentials of the solutions and the numbers of larvae which gained and lost weight are recorded in Table 10.

There is a significant correlation between the proportions of larvae losing weight and the water potential of the solution they floated on (Kendall's rank-order test; $P<0.025$). At all potentials of -900 J/kg or more, more larvae gained weight and at all

potentials of -960 J/kg or less, fewer larvae gained weight than lost it. The water potential at which the larvae would be in equilibrium is thus no different from the potential previously measured in haemolymph of third instar larvae (mean -867 J/kg; standard deviation 75 J/kg). Hence, under the conditions of these experiments water moved down potential gradients without any control by the larvae.

The previous experiments indicated that gain or loss of water from larvae in soil was limited, but gave no definite evidence for any controlling mechanism. The larvae floating on solutions lost weight about 40 times more rapidly than those in soil at the same water potential, which clearly demonstrates the insulating effect of an air layer around the cuticle. The surface properties which help to keep the larval cuticle dry would protect the larvae against this accelerated water movement except perhaps in saturated soil.

In both series of experiments, in soil and on solutions, the larvae survived considerable changes in water content. Second instar larvae survived the gain of up to 41% or loss of up to 54% of their initial weights. Third instar larvae did not tolerate such great changes - the largest weight gain survived was only 18% and the largest loss 38%. Although larvae probably would not survive such large changes for very long, these figures show that they can tolerate some water gain or loss if necessary.

Although the above experiments failed to demonstrate control of water balance in the larvae there must be a homeostatic mechanism effective under normal conditions since the variation of potentials measured from larval haemolymph was limited: the standard deviation was only about 10% of the mean of the measured potentials in each instar. Such homeostasis also occurs in other Scarabaeidae: Ludwig and Wugmeister (1953) found in their experiments that the osmotic pressure of the haemolymph of starved Popillia japonica larvae "remained constant throughout the entire starvation period". Internal processes were not studied, but some responses of C. zealandica larvae to water stress which might contribute to homeostasis were investigated.

Table 11. Percentage loss in weight of treated C. zealandica larvae during six hours at 75% humidity.

Percentage loss				
Treatment:				
control; live larvae	killed with KCN	killed with KCN; spiracles blocked	killed and lipid removed with chloroform	control; Agar blocks
8.4	11.7	8.7	43.2	36.4
11.3	12.5	10.2	45.2	44.1
11.7	14.0	12.0	53.5	47.0
12.0	14.7	14.6	55.7	48.8
13.7	15.3	15.1	58.2	49.2
14.5	16.0	15.5	60.3	54.3
14.7	16.5	15.8	60.5	57.2
15.7	19.9	15.9	64.0	67.1
18.7	20.6	16.0	65.4	
20.6	22.8	18.1	65.6	
21.2	24.6	20.0		
22.5	28.2	21.4		
23.7	31.0	23.7		
24.0	36.8	27.3		
24.5				
26.3				
27.5				
28.6				
31.1				
Mean percentage loss				
19.51	20.32	16.72	57.17	50.50

4.5.3 Water movement through the integument of the larva

One way of regulating water balance would be to control the rate of water movement through the integument. Changes in the permeability of the cuticle do not seem likely, but many insects control the rate of loss of water from the tracheal system by closing the spiracles and some also actively absorb water through the cuticle. No closing mechanism was found in the spiracles of C. zealandica larvae, nor has any been found in other scarabaeid larvae (Hinton, 1967). Just as there is no regulation of the diffusion of carbon dioxide and oxygen through scarabaeid spiracles (Le Berre and Hawlitzky, 1967) neither can there be any regulation of water vapour diffusion.

Movement of water through the cuticle and the spiracles of C. zealandica larvae were compared, and active control of water movement tested, by measuring transpiration from larvae treated in various ways. Transpiration rate was measured in 75% relative humidity using the method described in Section 1.6.6 except that air was bubbled through the saturated salt solution instead of stirring the air with a fan, and the larvae were left for 6 hours instead of 24. Normal, active third instar larvae were used as controls. To test whether transpiration was influenced by active processes linked with the animals' metabolism, larvae killed with cyanide were tested. To find how much water was lost through the spiracles, other larvae were killed with cyanide and their spiracles carefully blocked with spots of paint before being tested. To test how much the lipids of the epicuticle affected the transpiration rate, larvae treated with boiling chloroform for 5 minutes were also tested. Blocks of 2% agar gel cut to the size of a larva were also tested to give an approximation of evaporation from a free water surface. The percentage loss in weight from samples with all these treatments are given in Table 11. Differences between treatment effects were tested by Wilcoxon's Rank sum test.

If active processes had a significant effect on transpiration rate, dead larvae would lose water at a different rate from live ones. However their weight losses are not significantly different, so there

is no evidence for active processes. This is consistent with other results. Any active transport of water through the cuticle would affect the humidity in an enclosed space around a larva. Measurements by thermocouple psychrometer showed that this humidity was generally higher than the expected (equivalent to the water potential of the haemolymph), even over larvae desiccated by leaving them in the open air overnight. This suggests that larvae were losing water by thermo-osmosis (or possibly actively eliminating it). There is no evidence for the active absorption of water which occurs in other insects (Beament, 1961), probably through the cuticle of the rectum (Noble-Nesbitt, 1970). Drying of the faeces in the rectum by absorption of water has been demonstrated in some scarabaeid larvae (Czarnota, 1966; Sliwinski, 1966) but there is no evidence for this occurring in C. zealandica; larvae and their faeces had the same water potential, although rapid equilibrium in the period before the larva was removed for separate measurement may have eliminated any difference. Active transport is normally involved in the processes of absorption from the intestine, but this is outside the scope of this study.

Blocking the spiracles should lower the transpiration rate but the weight losses from larvae with blocked spiracles are not significantly less than those from normal larvae. Therefore the loss of water through the spiracles made up only a small part of the total water loss. A misconception about diffusion through small pores (such as the aeropyles of the larval spiracles) should be mentioned here. Buck (1962) considered that gas exchange through the apertures of insect spiracles would be "in proportion to their perimeters rather than to their areas", because of the "pore diffusion" effect of Brown and Escombe (1900). Brown and Escombe made the false assumption that the resistance to diffusion through pores was solely in the pores, and neglected the resistance to diffusion away from the pores. As Cowan and Milthorpe (1968) put it, "although the importance of the external resistance...has been pointed out by various workers over the years, repetition of the experiments - including the misinterpretations - has lead equally to the perpetuation of the misunderstandings and extension from them to absurd conclusions".

The resistance to diffusion through fine pores is not a simple function of pore dimensions.

The weight losses of normal and chloroform-treated larvae are so different that their ranges do not overlap at all; no statistical test is necessary to demonstrate the significance of the difference. Removal of lipid by hot chloroform trebled the transpiration rate of the larvae. The weight losses of chloroform-treated larvae and agar blocks are not significantly different. This does not mean that the integument of chloroform-treated larvae is as permeable as a free water surface (since evaporation from such permeable objects is 'vapour-limited') but does show that the lipid provides most of the resistance to water movement through the cuticle.

4.5.4 Behaviour of larvae in water potential gradients

The ability of C. zealandica larvae to keep their water potential constant would be improved if they avoided excessive water stress and moved to regions of more moderate water potential. Hence a series of experiments was designed to determine the responses of larvae to water potential gradients in soil.

Water potential gradients were set up by dividing containers in two with temporary partitions and filling each half with soil of a different percentage water content so that when the partition was removed there was an abrupt change in soil moisture (and hence in water potential) at the junction between the two halves. Soil of known percentage water content was made up by adding distilled water to sieved oven-dry soil and mixing it thoroughly in plastic bags. When the gradients were set up larvae were placed on the soil either in equal numbers on each side, or all along the centre line, and left to redistribute themselves over a period of two weeks. During this time the containers of soil were covered to reduce evaporation of water from the soil, and were kept in the dark so that responses to light would not influence the distribution of larvae. At the end of each experiment, the line of demarcation between the different soil moistures was still clear and the partition was re-inserted and the soil and larvae removed from each end separately. The numbers of larvae recovered from each end were

treated as a sample from a binomial population and tested for significant deviation from equality (i.e. no response) using cumulative binomial probabilities. Third instar larvae were used in most of the experiments but first and second instar larvae were also tested.

In the first experiment, third instar larvae were tested using 'wet' soil of 14% water content (about -700 J/kg water potential) and 'dry' soil of 6% water content (about -8500 J/kg water potential). The numbers recovered from each end were:

Wet	Dry
120	36

Under the null hypothesis that larvae did not respond to the difference in water potential and were thus equally likely to be in either end, the probability of 120 or more being in the wet end is much less than 0.0001. Hence the alternative hypothesis is accepted: the larvae did respond to the difference in water potential and tended to remain in the wetter end.

To find whether sense organs on the antennae were involved in this response the experiment was repeated on larvae with their antennae removed. The antennae were cut through the basal segment close to the cranium with fine scissors and the larvae left in soil for several days. Those which remained active and appeared healthy were used, with normal larvae as controls. Under the same conditions as before, the numbers of larvae recovered from each end were:

	Wet	Dry	Probability
Normal:	83	19	$P < 0.0001$
Antennae removed:	27	30	$P > 0.50$

While normal larvae moved to the 'wet' side, larvae without antennae did not, which suggests that sense organs on the antennae are involved in the response. However the lack of response of these larvae may be merely the result of their mutilation. To test this, larvae with their maxillary palps removed instead were tested. Also in this experiment other larvae with only the last segment of each antenna removed were tested, since most of the sense organs found on

the antennae were on this segment. Under the same conditions as before the distributions of larvae were:

	Wet	Dry	Probability
Normal:	23	2	$P < 0.0001$
Maxillary palp removed:	37	11	$P < 0.0005$
Antennae removed:	14	9	$P = 0.25$
Last antennal segment removed:	34	13	$P < 0.005$

Once again normal larvae moved to the 'wet' side, and so did the larvae with their maxillary palps removed, so mutilation alone did not stop the response. Again, larvae with no antennae failed to respond, although those with only the last segment missing did respond. This supports the hypothesis that sense organs involved in this response are on the antennae, and suggests that they are not restricted to the last segment. This eliminates several of the types of sense organs found on the antennae, but there is not enough information to speculate on which type actually is involved.

Unfortunately this particular series of experiments was abruptly terminated when normal larvae stopped responding to the moisture gradients. This change in behaviour was quite sudden as can be seen from Table 12 in which the distributions of normal larvae in the experiments already discussed and two further ones are listed, with the dates the experiments were completed and the (one-tailed) probabilities of these distributions occurring at random.

Table 12. Distributions of third instar C. zealandica larvae in water potential gradient experiments, showing their change in response.

Date completed	Number in 'wet' side,	'dry'	Probability
16-7-69	120	36	$P<0.0005$
21-7-69	83	19	$P<0.0005$
30-7-69	23	2	$P<0.0005$
11-8-69	18	20	$P>0.25$
19-8-69	13	8	$P>0.25$

Table 13. Positions of third instar C. zealandica larvae in water potential gradients.

Gradient				Position of larva (W=wetter, D=drier)			
% water;		potential J/kg		Initially in left;		in right	
left	right	left	right	12 hrs	24 hrs	12 hrs	24 hrs
4	8	-20,000	-4,200	W	D	W	-
6	8	-8,500	-4,200	D	W	-	-
7	8	-6,000	-4,200	-	W	D	-
9	8	-3,000	-4,200	W	W	W	-
10	8	-2,400	-4,200	-	-	W	W
12	8	-1,300	-4,200	-	-	W	W
16	8	-320	-4,200	W	D	W	W
20	8	-110	-4,200	W	W	W	-
24	8	-60	-4,200	W	W	W	W
28	8	-35	-4,200	-	-	W	W
4	6	-20,000	-8,500	D	W	D	-
6	7	-8,500	-6,000	W	W	W	W
7	8	-6,000	-4,200	D	W	W	D
8	9	-4,200	-3,000	W	D	D	-
9	10	-3,000	-2,400	W	-	W	W
10	12	-2,400	-1,300	W	W	W	D
12	16	-1,300	-320	W	W	W	W
16	20	-320	-110	W	W	W	W
20	24	-110	-60	W	D	W	W
24	28	-60	-35	D	W	W	W
4	12	-20,000	-1,300	W	W	W	W
6	12	-8,500	-1,300	D	W	W	W
24	12	-60	-1,300	W	W	W	W

All these larvae were collected from West Melton a few days before being used, and stored in soil at 7°C.

The change in response may be a physiological change occurring in the 'prepupal' period prior to pupation. This is not a clearly defined stage but in scarabaeid larvae normally refers to the period "characterised by internal activity, a cessation of feeding, some body shrinkage, and the cleaning out of the alimentary canal" before pupation (Hayes, 1929). Perrott, Shorland and Czochanska (1965) recorded that weight and fat content of third instar C. zealandica larvae began to decline at the beginning of September, so they probably cease feeding a short time before this - about the time of the above change.

The responses of third instar larvae to a range of water potential gradients was studied in another series of experiments (which was also interrupted when the larvae stopped responding near the end of July). In these experiments, the containers of soil were clear plastic petri dishes and only one larva was placed in each, so that its movements could be followed. Gradients of water potential were set up as before. Larvae were placed on one side of each and their positions marked on the plastic tops of the dishes before they were all covered to keep out the light. After 12 and 24 hours they were uncovered and the positions of the larvae marked. To give a replicate all the larvae were then removed and redistributed among the dishes at random, placed in the opposite side of the dish from that used in the first run, and the whole experiment repeated. The soil water contents used in the gradients (with their approximate water potentials) and the positions occupied by the larvae at each observation (classified as being in the wetter (W) or drier (D) side) are listed in Table 13, except where the larva had not moved between successive observations.

If the larvae prefer soil with a water potential close to their own, they would move to soils with water potentials near -800 to -1000 J/kg (water content 12-16%). Where both soils in the gradients had 12% or less water, 33 out of 43 larvae moved to the wetter soil as expected, but where both soils had 16% or more water still 10 out of 12 larvae moved to the wetter soil. Under the null hypothesis that

larvae were equally likely to move to either end, the probabilities of such high numbers being in the wetter end are 0.0005 and 0.025 respectively. Hence it appears that the larvae moved to the wetter end in both cases. The thermocouple psychrometer measurements of the water potential of larvae such as these were higher (i.e. 'wetter') than the expected equilibrium value, probably because of continual loss of water by thermo-osmosis, so larvae seeking a soil water potential where they would neither lose nor gain water (in this case a condition of steady state rather than equilibrium) would move into wetter soil as in these experiments. Grouping all the results, in 76 observations, the larvae were in the wetter end 62 times. The probability of there being this many in the wetter end (under the null hypothesis of no response) is much less than 0.0001. Thus the larvae showed no preference for any particular range of water potential among those used, but generally moved into the wetter soil, unlike larvae of Sericesthis geminata (Melolonthinae) which have "been shown to move rapidly into soil with a 15% moisture content, when exposed to moisture gradients from 5% to 30%" (Davidson, 1969).

To find whether the larvae responded to small gradients as well as larger ones the results from Table 13 are classified in Table 14 according to the magnitude of the potential gradient, measured by the percentage change in estimated water potential from the 'dry' to the 'wet' side. The class limits were chosen arbitrarily to give similar numbers in each group.

Table 14. Responses of third instar C. zealandica larvae to different water potential gradients.

% water potential change	larvae in:		probability
	wet	dry	(H_o:no response)
less than 50%	19	6	0.01
50-70%	15	4	0.01
70-95%	16	3	0.005
over 95%	13	0	0.0005

The response was greater in the steeper gradients (large percentage water potential change) but was still significant where the potential changed by less than 50% across the gradient. These were mainly where the difference in percentage water content was only 1%, so the larvae responded to the smallest water potential gradients used.

In a further series of experiments with water potential gradients in soil in plastic petri dishes, larvae were observed half-hourly. Their tracks through the soil were usually visible, and were marked on the lids of the dishes. These tracks were examined for evidence of the behaviour patterns described by Fraenkel and Gunn (1940) which might orient the larvae in the gradients. There was little evidence of directed reactions, as might be expected since the gradients are not very steep compared with the size of the animals. However the distances moved by the larvae appeared to vary with the water potential of the soil. To test this, the distances moved by the larvae in each 30 minute period were examined. Often the larvae moved only a short distance or not at all, so to avoid uncertain and subjective measurements the maximum distances only were measured. The maximum distance moved in 30 minutes by larvae in each soil water content are listed in Table 15. Movements where larvae crossed into the other side of the gradient are excluded.

Table 15. Rate of movement of third instar C. zealandica larvae in different soil water contents.

% water content	4	6	7	8	9	10	12	16	20	24	28
rate of movement	5.6	8.0	4.0	3.6	5.8	3.8	2.6	1.8	3.4	2.0	1.0

Rate of movement measured as maximum distance (in cm) travelled in 30 minutes.

The maximum rates of movement show a highly significant correlation with soil water content, and hence with soil water potential (Kendall's

rank correlation test: $P<0.005$). Fraenkel and Gunn (1940) called this behaviour pattern, in which rate of movement depends on the intensity of a stimulus, orthokinesis.

The responses of the first and second instar larvae to water potential gradients were tested in the same way as those of third instar larvae, using a range of gradients with 20 or more larvae in each. The results are listed in Table 16 with their chi-squared values and corresponding probabilities calculated under the null hypothesis of random distribution.

Table 16. Responses of first and second instar C. zealandica larvae to water potential gradients.

First instar larvae:

% soil water in drier end	7	7	7	7	15	15	
in wetter end	22	15	12	12	22	22	
							totals
larvae in drier end	18	8	13	13	10	11	73
larvae in wetter end	20	11	6	6	9	7	59

($\chi^2 = 6.68$ Probability > 0.25)

Second instar larvae:

% soil water in drier end	0	4	8	16	20	24	28	
in wetter end	4	8	12	20	24	28	32	
								totals
larvae in drier end	7	7	11	11	12	7	7	62
larvae in wetter end	12	13	8	7	8	13	13	74

($\chi^2 = 8.88$ Probability > 0.10)

% soil water in drier end	4	8	12	16	16	16	16	
in wetter end	16	16	16	20	24	28	32	
								totals
larvae in drier end	9	12	24	11	8	10	7	81
larvae in wetter end	9	7	35	18	10	8	12	99

($\chi^2 = 6.82$ Probability > 0.25)

The null hypothesis of random distribution with respect to water potential can not be rejected from any of these sets of results; the larvae showed no preference for any water potential. The total numbers in the 'wet' and 'dry' ends, are not significantly different: the cumulative binomial probabilities of 73 or more out of 132 first instar or 143 or more out of 316 second instar larvae being in one end by chance are both over 0.1. Therefore these larvae did not move to the wetter soil as the third instar larvae did. The results presented in Section 2.3 showed that second instar larvae in water potential gradients congregated near food (growing roots) no matter which side of the gradient it was on. The same sense organs were found on all the larval instars so although first and second instar larvae did not respond to water potential gradients in these experiments, they are probably capable of doing so. Kelsey (1968) observed that larvae, including first instar larvae moved closer to the surface when dry soil was artificially watered.

4.6 Water stress and the pupa

The pupa lives for only a short time in its cell in the soil before emerging as an adult beetle. In an attempt to find out just how long, larvae were placed in soil between sheets of glass and the dates when the visible ones pupated were recorded. Most did not develop properly, or were killed when other larvae burrowed into their cells, but two developed and successfully emerged as beetles. These larvae pupated 8 and 11 days after they ceased moving through the soil, and emerged as adults 15 and 12 days later: a total time of 23 days in each case. One beetle failed to reach the surface, but the other waited in the pupal cell for 8 days, then took 4 days to burrow 17 cm to the surface and emerge.

While in the pupal cell the animal is unable to avoid the water stress imposed by the conditions in that region of the soil. Thus the choice made by the prepupal third instar larva of a place to construct the cell and pupate has an important bearing on the stress undergone by the pupa and its consequent chance of survival. Conditions in the soil near the surface change more rapidly than in

the deeper soil, where conditions are relatively moderate, and the pupa is likely to be under lower stress if the prepupal larva burrows down in the soil to pupate, even if at the time the region near the surface is temporarily more suitable. Behaviour patterns having this result (a tendency to burrow downwards, and failure to respond to water potential gradients in the soil) have already been described in prepupal larvae. As a result the larvae build their cells and pupate deeper in the soil than where they were feeding whether the soil is wetter there or not.

As well are being immobile, and thus unable to avoid water stress, the pupa does not feed and thus loses a potential source of water in the food. Water potential measurements from the body fluid of pupae (Table 6; facing page 59) had the lowest standard deviation of any of the stages of C. zealandica. This indicates efficient control of water balance, which is surprising considering the turmoil of physiological change in the pupa as adult tissues are substituted for larval ones.

During the period when C. zealandica is in the pupal stage (October-November), the soil at West Melton is normally dry so the pupa will tend to lose rather than gain water. Its cuticle is not required to withstand abrasion by soil particles as the larval cuticle is, and it is only half as thick as the larval cuticle. In the transpiration rate experiments, pupae lost water as rapidly as active larvae, so the pupal cuticle has no additional properties restricting water loss. No closing mechanism was found in the spiracles of the pupa, but as in the larva, loss through the spiracles is unlikely to make up much of the total water loss.

Thermocouple psychrometer measurements of the water potential around a pupa fluctuated between -1200 and -1400 J/kg while it was alive, but when it died its water potential rose to a level varying about -940 J/kg, which is within the range measured in pupae by the freezing point depression technique. There is a possibility that this difference in water potential of the pupa is produced by active absorption of water, for it is in the opposite direction to that which would be produced by thermo-osmosis associated with the heat flow from a respiring pupa.

The contact angles of water measured on the pupa were similar to those measured on the other stages as might be expected from the similar surface structure and composition of their cuticles. These contact angles are high enough to retard the spreading of liquid water over the surface of the cuticle. The pupa lying in its smooth-walled cell rests mainly on narrow dorsal ridges (fig. 63) and is thus further protected against contact with liquid water in the soil. The comparatively wide air space around the pupa in its cell provides considerable resistance to diffusion of water, and the smooth cell walls minimise the area available for diffusion into the soil. As the thin sections and SEM photographs showed, the cell wall is formed by compaction of the soil, with no further modification. For the few weeks that the pupa occupies the cell until the beetle emerges, the resistance in the air space is probably sufficient to prevent excessive water loss even without any further 'waterproofing' of the cell by special treatment of its walls.

4.7 Responses of the adult beetle to water stress

4.7.1 Effects of water stress on the beetle's water content, longevity and oviposition

The responses of adult C. zealandica beetles to water stress were investigated in a series of laboratory experiments. The first was designed to determine the effects of water stress on water content, longevity and oviposition, by comparing beetles under conditions of low and high water stress. Four jars of soil were prepared, two with wet and two with dry soil. Their actual water contents were determined at the end of the experiment. Five male and five female beetles (collected from the soil at West Melton before emerging from the ground) were placed in each jar and another jar inverted over it to confine the beetles and reduce evaporation of water from the soil. Each day the soil from each jar was sieved, eggs and dead beetles removed and counted, and water added to the wet soil. The dead beetles were desiccated at 105°C to find their water contents. The experiment was terminated after 25 days when no eggs had been found for four consecutive days and only 7 of the original

40 beetles were still alive. The water content of the soil in each jar was then determined. Results and discussion of each section of this experiment are presented separately.

The soil moistures were not controlled but maintained at widely differing water potentials. At the end of the experiment, the 'wet' soils contained 19.87% and 19.40% water which correspond to water potentials near -110 J/kg, and the 'dry' soils contained 7.38% and 7.59% water - water potentials near -5000 J/kg.

The mean weight and water content of the beetles in this experiment are given in Table 17 with figures from teneral beetles (collected from their pupal cells at West Melton) for comparison.

Table 17. Weight and water content of C. zealandica beetles.

	Mean weight	Percentage water content	
	mg	mean	standard deviation
Teneral beetles	106.0	65.0	
Beetles in 'dry' soil			
at start	90.5		
at death	50.3	67.0	2.82
Beetles in 'wet' soil			
at start	83.4		
at death	49.5	71.6	2.00

Water potential was higher in the wet soil and considerably lower in the dry soil than the water potential measured in haemolymph of beetles, hence beetles in the wet soil would tend to gain water and those in the dry soil to lose it. The results show however that even at death, beetles in both soils still had percentage water contents similar to that of teneral beetles even though they lost over half their initial weight as they used up their fat reserves. Beetles in the dry soil lost slightly more weight and contained less water at death than those in the wet soil, but the difference is not very great. This indicates that despite the water stress imposed by their environments, the beetles maintained their water contents at a

constant high level. The measurements of water potential of beetle haemolymph given in Table 6 (facing page 59) were also within a restricted range (mean -1007, standard deviation 123 J/kg) and showed no tendency to change with the weight or age of the beetles. Thus both the water content and water potential of the beetles are independent of changes in the environment or the condition of the beetles, which are therefore not in equilibrium with the water in the environment but maintain their water balance in a steady state. Once the eggs are laid the fate of the adult beetle is of no consequence for the survival of the species and continued control of its water balance appears to have no further advantage.

The numbers of eggs the beetles laid each day in the 'wet' and 'dry' soils in this experiment are listed in Table 18.

Table 18. Eggs laid by C. zealandica beetles in wet and dry soil.

Days from start	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Wet soil (1) 86 eggs laid:	2			31	26	1	5	19		1	1				
Wet soil (2) 79 eggs laid:				40	1		26	11				1			
Dry soil (1) 92 eggs laid:		25		3	8		8	11	9	22	6				
Dry soil (2) 71 eggs laid:							21	1	47						2

The proportions of eggs laid up to day 13 (when half of all the eggs had been laid) were:

	'Wet'	'Dry'	Total
Eggs laid up to day 13	65, 67	44, 21	197
after day 13	21, 12	48, 50	131

First, comparing the proportions in each treatment, there is no significant difference between the proportions in the two 'wet' soils ($\chi^2 = 0.44$, 1 d.f., $P>0.25$), or in the two 'dry' soils ($\chi^2 = 3.35$, 1 d.f., $P>0.05$). However there is a highly significant

difference between the proportions in all four (χ^2 = 24.8, 3 d.f., P<0.005); beetles in the 'wet' soil laid their eggs earlier than those in the 'dry' soil.

The beetles in this experiment laid fewer than half the number of eggs recorded by Kelsey (1951) or Fenemore (1965). Despite the differences in water potential and hence in water stress on the beetles, the numbers of eggs laid in the four jars were not significantly different (χ^2 = 3.0, 3 d.f., P>0.25). Hence the water stress on the beetles had little if any effect on the number of eggs they laid.

Other scarabaeid beetles lay fewer eggs at extreme water potentials: Maelzer (1961) stated that Aphodius tasmaniae laid most eggs in soil of pF 2.8-3.2 (about -65 to -160 J/kg water potential) which was also the range in which they aggregated, and where survival was optimal. Sweetman (1931) found that in Phyllophaga implicita "oviposition is sharply limited at the high and low percentages of saturation". His figures can not be converted to water potentials.

The time each beetle in this experiment survived is given in Table 19.

Table 19. Longevity of C. zealandica beetles in wet and dry soil.

Time of survival in days from beginning of experiment:

Female beetles:

Wet soil:	7,	13,	13,	16,	18,	19,	20,	24,	25+,	25+.
Dry soil:	12,	15,	18,	18,	19,	19,	22,	24,	24,	24.

Male beetles:

Wet soil:	3,	6,	11,	14,	14,	14,	18,	20,	25+,	25+.
Dry soil:	10,	13,	15,	18,	18,	19,	20,	24,	24,	25+.

By the 19th day, when half of all the female beetles had died, there were four surviving females in each treatment. Wilcoxon's rank sum test applied to all the female beetles, ranked by the days they survived, gives a probability of more than 0.50 that survival was no different in the 'wet' and 'dry' soils. Similarly it can be shown

Table 20. Distribution of C. zealandica beetles in soil water content choice experiments.

Soil water content:	7%	12%	15%	22%
(1) 13/11 - 17/11 (eggs found in all but 7%)				
males	0	23	30	10
females	1	12	23	10
total	1	35	53	20
(2) 17/11 - 18/11 (no eggs found)				
males	23	21	30	49
females	67	59	105	205
total	90	80	135	254
(3) 17/11 - 20/11 (continuation of (2))				
males	1	27	19	41
females	3	105	67	112
total	4	132	86	153
eggs	21	200	124	175
Totals of (1), (2) and (3)				
males	24	71	79	100
females	71	176	195	327
total	95	247	274	427

that there was no difference in survival of male beetles.

Thus water stress did not affect survival of the beetles in this experiment, but female beetles under stress delayed laying their eggs. A similar effect was observed in Phyllophaga (Melolonthinae) beetles by Sweetman (1927). This suggests that the female beetles are sensitive to water potential and may be able to choose regions of lower stress for laying eggs. The following series of experiments were carried out to test for this type of behaviour.

4.7.2 Water potential preference of beetles burrowing into soil

The second series of experiments was designed to determine whether beetles preferred to burrow into soil of any particular water potential. The bottom 10 cm of a round tin 20 cm in diameter and 25 cm high was divided into four sectors by cardboard partitions. These sectors were filled in a random order with soil of 7, 12, 15 and 22% water content (about -6000, -1300, -475 and -80 J/kg water potential respectively) level with the top of the partitions. Beetles were collected off the ground at West Melton during and after an evening flight and taken back to the laboratory. They were scattered over the surface of the soil and the tin covered with clear plastic and left for several days so that they could emerge and burrow into the soil again. Then the soil in each sector was scooped out and beetles and eggs sieved out and counted. Beetles on the surface were discarded. The results are listed in Table 20.

There is no significant difference between the distributions of male and female beetles in experiment (1) (χ^2 = 1.08, 2 d.f., P>0.5), experiment (2) (χ^2 = 2.60, 3 d.f., P>0.25) or experiment (3) (χ^2 = 1.66, 2 d.f., P>0.10). Although the distributions of beetles among the four soil water potentials are slightly different in the three experiments the overall trend shown in the grand totals is for greater numbers in the wetter soils.

Beetles appeared to burrow into the soil as soon as they were placed on it. In experiment (2) they were sieved out after one night, replaced, and sieved out again after two more nights (experiment (3)). Thus in (1) and (3) beetles could have emerged normally and burrowed into the soil again during several nights, and in these experiments

Table 21. Distribution of *C. zealandica* eggs and female beetles in different soil water contents.

% water content	in top 5 cm	22	15	12	7	22	15	
	in bottom 5 cm	7	7	7	12	15	22	
								Totals
eggs	in top 5 cm	3	2	4	11	0	1	20
	in bottom 5 cm	86	71	66	88	101	65	447
female beetles	in top 5 cm	8	10	9	9	9	8	53
	in bottom 5 cm	2	0	1	1	1	2	7

there were very few beetles in the soil with only 7% water. Beetles must have moved out of this soil between the counts of (2) and (3) for the number was 20 times smaller at the second count. Thus even when they were very crowded, the beetles avoided the driest soil and moved into the wetter soils.

The distribution of eggs among the four soils was similar to that of the beetles but when the number of eggs laid per female beetle present are considered there are actually more in the soil with 7% water than the others. This may be because this soil was much less crowded with beetles. At any rate there is no evidence from these experiments that egg-laying females react any differently to water stress from the other beetles; they all avoid burrowing into the drier soil.

4.7.3 Behaviour of female beetles in water potential gradients when laying eggs

The third series of experiments was designed to find whether soil water potential influenced the depth at which female beetles laid their eggs. Plastic containers 5 cm deep were filled with soil of one water content and similar containers with their floors cut out were placed on the first and filled with soil of another water content. Ten female and three male beetles (chosen for their activity from the beetles recovered from experiment (3) of the previous series) were placed on the surface of the soil in each, and a further container inverted over the top to confine the beetles and reduce evaporation from the soil. After seven days the different soils were separated by sliding a thin metal sheet between the bottom two containers and the eggs and beetles sieved out and counted. The beetles were replaced for a further seven days but no eggs were found when the soil was sieved again. The results are listed in Table 21.

The last four combinations of soil water contents are matched pairs of the combinations 7%/12% and 15%/22%. The numbers of eggs and beetles found in these can be used to test alternative hypotheses on the proportions in each half:

(a) there are more in the wetter half
(b) there are more in the drier half
(c) there are more in the top half
(d) there are more in the bottom half
(e) there is the same number in each half

The total numbers of eggs, and of female beetles in the halves are:

Eggs:	top	16	dry	179
	bottom	320	wet	157
	total	336		336

Female beetles:

	top	35	dry	19
	bottom	5	wet	21
	total	40		40

Since the categories in each pair are mutually exclusive the probabilities of these proportions can be calculated from the binomial distribution.

Under the null hypothesis (e), the probability of there being 179 or more of the 336 eggs in the drier half is greater than 0.25, hence (e) can not be rejected in favour of hypothesis (b). Hypothesis (a) is even less likely to be true.

Under the null hypothesis (e), the probability of there being 320 or more of the 336 eggs in the bottom half is much less than 0.0005, hence (e) can be rejected in favour of hypothesis (d).

Thus eggs were laid near the bottom whether it was wetter than the top half or not.

Under the null hypothesis (e), the probability of there being 21 or more of the 40 female beetles in the wetter half is greater than 0.25, hence (e) can not be rejected in favour of hypothesis (a). Hypothesis (b) is even less likely to be true.

Under the null hypothesis (e), the probability of there being 35 or more of the 40 female beetles in the top half is less than 0.005, hence (e) can be rejected in favour of hypothesis (c).

Thus female beetles (after laying their eggs) burrowed to the top half whether it was the wetter half or not.

These experiments show that the female beetles laying eggs burrow downwards, in spite of water potential gradients, just as the prepupal larvae did. In both cases the succeeding immobile stage (egg or pupa) is placed deeper in the soil where water stress is likely to be lower, even when conditions happen to be temporarily more favourable nearer the surface at the time the active beetle or larva is choosing the site. After laying the eggs, the female beetle burrows back to the surface.

Of the six trials in this experiment, three had soil with 7% water (-6000 J/kg water potential) and three had wetter soil in the bottom half where most of the eggs were laid. There is no significant difference between the numbers of eggs laid in these two subsamples (Wilcoxon's rank sum test: $P>0.2$), hence in this experiment also, the water potentials used had little or no effect on the number of eggs laid, although again these numbers were small.

4.8 Water stress and the egg

4.8.1 The effect of water stress on hatching success

The effect of water stress on hatching success of eggs was tested in the following experiment. Eight small containers were filled nearly to the top with sieved soil of 5, 7, 9, 11, 13, 15, 18 and 22% water content (about -12000, -6000, -3000, -1800, -1000, -500, -200 and -0.8 J/kg water potential respectively). Fifteen unswollen eggs (laid by beetles in a previous experiment) were scattered over the soil in each container and covered with more soil. The containers were covered to prevent loss of water from the soil. Three weeks later the soil from each container was sieved and all larvae counted. Unhatched eggs were replaced for a further week but none hatched. From fifteen eggs in each soil, the following numbers hatched:

% water in soil:	5	7	9	11	13	15	18	22
larvae hatched:	2	14	14	11	14	15	15	15

Most eggs hatched successfully in all but the driest soil. Since the water potential measured in C. zealandica eggs was normally above -1000 J/kg, the eggs which hatched in the soil of 5% water

content survived the water stress from a difference in water potential of about 10,000 J/kg acting to remove water from them. Under lower water stress - due to differences in water potential of 5,000 J/kg or less, nearly all the eggs hatched. Eggs also developed and hatched floating on distilled water - an environment of zero water potential imposing water stress acting in the reverse direction to that in the above experiment.

4.8.2 Active absorption of water by the egg

It has already been shown that the eggs are relatively permeable to water and that they absorb water rapidly during one phase of their development. Even the eggs which hatched in the drier soils were observed to swell during development. The above results show that they develop and hatch even under considerable water stress. This suggests that they are capable of absorbing water against a potential gradient: active transport by Rosenberg's (1954) criterion. Further evidence supports this hypothesis.

In the transpiration rate experiment, eggs lost 40% of their weight in 24 hours at 75% relative humidity - a water potential of -40,000 J/kg. Eggs developing on wet filter paper with a water potential close to 0 J/kg gained up to 73% in weight in 24 hours. Water potentials measured in eggs were near -1000 J/kg so in terms of the gradients in each case, the eggs absorbed water several hundred times faster than they lost it. Active absorption of water by the eggs could possibly contribute to this effect.

Active transport uses metabolic energy, probably from ATP, or directly from electron transfer in the respiratory chain (Whittam, 1964). In eggs of Popillia japonica (Scarabaeidae), oxygen consumption rises and activity of the enzymes in the respiratory chain is high during the water absorption phase (Ludwig and Wugmeister, 1955). If the respiratory metabolism is involved in energy production for active transport, poisoning the enzyme system will stop water absorption. Therefore the effect of cyanide, which inhibits many enzymes including some in the respiratory chain (Dixon and Webb, 1964) was briefly examined.

Newly laid C. zealandica eggs placed on filter paper soaked in potassium cyanide solution did not swell normally. Instead, the part of the egg in contact with the cyanide solution collapsed and flattened against the paper while the rest of the egg remained rounded but soft. This suggests that absorption of water into the egg is an active process, controlled by an enzyme system.

Experiments on development and hatching success of eggs of other scarabaeids have given different results. Laughlin (1957) found that Phyllopertha horticola eggs gained weight when floating on sucrose solutions with water potentials (given as osmotic pressures) greater than -1770 J/kg, but lost weight on solutions of -2020 J/kg or less. His measurements of the potential of the egg contents ranged from -740 to -1720 J/kg. Maelzer (1961) investigated the changes in weight and hatching success of Aphodius tasmaniae eggs in soils of known water potential (given in pF values). He found that the eggs absorbed water and hatched normally in potentials from -30 to -560 J/kg but lost water and failed to hatch at -1000 J/kg water potential. In neither species is there absorption of water or hatching at water potentials significantly below that of the eggs; there is no evidence for active transport of water against potential gradients.

4.8.3 Absorption of water vapour by the egg

Kelsey (1951) stated (of C. zealandica eggs) that "for development they require to be in contact with moisture". Liquid water in porous materials like soil retreats into smaller pores and crevices as the material dries, and at a potential of -1500 J/kg (which is common during summer) fills only the pores less than 0.2 μm across. Thus contact of the eggs with liquid water must be very limited, although the colleterial secretion on the eggs could increase it by attaching the eggs more closely to the surrounding soil as well as by surface active effects. The situation is very similar in plant roots which absorb water from soil; these are surrounded by mucilaginous material which "fills the space between cell wall and mineral soil particle" (Jenny and Grossenbacher, 1963).

To test whether C. zealandica eggs could absorb water vapour from the air when not in contact with liquid water they were placed on a dry surface in a closed container with filter paper soaked with water to maintain a high humidity. The dry surface was a glass slide treated with dimethyldichlorosilane to make it water repellent (Holland, 1964). Under these conditions three newly laid eggs swelled and increased to 175% of their initial weight after 8 days, and hatched after 16 days. Hence the eggs can absorb water and develop normally without contact with liquid water.

The rate of diffusion of water vapour through air depends on the vapour pressure gradient, which is related to the water potential gradient. Cowan and Milthorpe (1968) gave a relationship between the gradient of water potential and rate of flow of water vapour across an air space in their discussion of absorption of water by plant roots in soil. From this relation it can be shown that for a developing C. zealandica egg to absorb water from the air as rapidly as from wet filter paper, the water potential at the egg's surface, whether lowered by active absorption or not, must be 1,900 J/kg lower than that in the adjacent soil for each millimeter of air space separating them. As the female beetle lays the eggs in close contact with the soil, rather than in cells as do many other Scarabaeidae, the air space around the egg is kept small, especially as the eggs swell during development.

During summer when the eggs are present the soil water potential is normally below -1,500 J/kg, but the egg absorbs the water it needs even under these conditions. Like all the stages of C. zealandica it is capable of withstanding greater water stress than it normally encounters.

CONCLUDING REVIEW

Water is an important part of the environment of any animal. In this study the place of water in the life of a soil animal, the grass grub Costelytra zealandica (White) has been investigated. The morphology, behaviour and physiology of C. zealandica are similar to those of other scarabaeid species around the world, particularly those of the tribe Sericini. Most published work does not permit comparison of their performance under water stress, but C. zealandica appears to be particularly resistant to water stress, especially in the egg stage.

When studying water relations of most animals it can be assumed that water stress is invariably in the same 'direction': freshwater animals are exposed to too much water and continually gain it; marine and open air terrestrial animals are exposed to too little and continually lose it. However animals living in soil (or in an estuary) are exposed to too much water at some times and too little at others, so a way is needed to determine the direction as well as the magnitude of the water stress on these animals at any time.

The idea, that an animal is under water stress whenever the amount of water in its environment is unfavourable to optimum growth, implies that when there is either more or less water in the environment than some optimum amount, the animal will suffer undesirable change in water content unless it works to overcome this. Work is needed to oppose the physical forces promoting diffusion of water between the animal and its environment, and may be expressed in terms of a thermodynamic quantity: the chemical potential of the water. Thus the water stress on the animal, determined by the direction and force of passive diffusion between animal and environment, may be expressed and measured in physical terms.

Inadequate measures of the water in soil have restricted work on water relations of soil animals. The chief advantage of the percentage weight of water in soil is its simplicity of measurement, while converting it to a percentage of the amount of water needed to saturate the soil does not entirely remove its dependence on soil type.

The pF measure is much more realistic but does not take into account all the forces on the water in the soil. Expressing the state of the water in terms of its equilibrium humidity is theoretically sound but the high values normally encountered in soil invite a misleading approximation to 100% humidity and the false conclusion that soil is like a fresh-water environment. A better measure is the water potential, which is a practical form of the chemical potential of water, related to the traditional measures of the state of water in systems: hydrostatic, osmotic and matric pressures, relative humidity, etc. In an isothermal system water diffuses spontaneously from higher to lower water potential and the work involved is proportional to the change in potential and the amount of water moved, apart from 'frictional' losses.

The usefulness of the thermodynamic approach has been demonstrated but it must be left for future work to develop and refine it, and to exploit it to maximum advantage. Since soil animals tend to lose water at some times and gain it at others, they may be expected to incorporate some of the abilities both of the terrestrial animals which conserve water and of the freshwater aquatic animals which eliminate it. Further study could show just how soil animals differ from other animals, particularly in the way they keep their water in a steady state.

C. zealandica spends the greatest part of its life cycle as a larva, mainly in the third instar. The soft and helpless appearance of larvae removed from the soil is deceptive, for in soil they are well adapted to withstand the rigours of their environment. They move freely through the close-packed, abrasive soil by digging with their heavily armoured mandibles. They do not burrow aimlessly, but gather near roots to feed. As the greatest concentration of roots in pasture is near the surface the larvae are found there too, but they avoid the light and so normally remain underground.

The larvae normally maintain their water potential near -800 J/kg while the water potential of their soil environment varies from less than -1,500 J/kg in dry weather, to nearly 0 J/kg in wet weather. The consequent water stress on the larvae is small by

comparison with that borne by insects living in the open air, but the latter insects can not tolerate the reversal of the direction of stress that occurs in soil. The actual physiological regulating mechanisms which maintain internal water balance in the larva and the other stages have not been investigated; this must be left for future work.

Although the variations in water content and water potential of C. zealandica larvae are normally quite small, the larvae can tolerate considerable gain or loss of water in extreme conditions. Water movement through their cuticle is influenced by many properties and processes but no active control was found. The temperature gradient produced by the heat of respiration of the larva causes movement of water out through the cuticle by thermo-osmosis, but this process can hardly be said to be under the control of the larva. Continual uncontrolled loss of water by thermo-osmosis may possibly produce a net loss of water even in conditions of excess water. The thin soft cuticle of the larva is typical insect cuticle with most of the resistance to water movement in the lipid layers of the epicuticle, so abrasion by soil considerably increases its permeability. Unless the cuticle is wet, which the high contact angles of water on the cuticle help to avoid, movement of water through the cuticle is limited, not by the cuticle itself, but by the air around it, where diffusion of water vapour is comparatively slow. Thus the space which the larva digs around itself as it burrows through the soil helps to insulate it against rapid gain or loss of water.

As water moves through the cuticle so rapidly, the amount which passes through the spiracles is insignificant, so the lack of any closing mechanism on the spiracles is no disadvantage. Closing of the fine openings (aeropyles) in the spiracles by flooding with water is prevented by the high contact angles on their cuticle. This does not apply to first instar larvae whose spiracles have no openings at all.

Active third instar larvae have sense organs on their antennae which can detect quite small changes of water potential in the soil, so the larvae can move to avoid water stress. Further investigation is needed to identify the particular sense organs involved and to find

how they operate. The larvae burrow more rapidly in drier soil and generally move to the wetter end of water potential gradients. First and second instar larvae have the same sense organs, so are probably also sensitive to water potential gradients, but in laboratory experiments they did not respond to such gradients, and moved against them to find food. Second instar larvae are particularly resistant to water stress and tolerate even greater changes of water content than do third instar larvae.

As the third instar larva approaches pupation in spring, many changes occur in its physiology and behaviour. It stops feeding and moves downward in the soil, no longer responding to water potential gradients, and then builds a smooth-walled oval cell in which it pupates. The cell is formed by the larva pressing against the soil, and has no special lining. In its cell the pupa rests on its dorsal ridges with a clear space all around it. This air space protects it against rapid gain or loss of water during the few weeks before ecdysis: even though the pupa is the most permeable stage, its water potential varies least.

A few days after ecdysis the adult beetle burrows out of the pupal cell and up to the surface of the soil. It emerges from the soil briefly at dusk for mating and other activities and then shelters in the soil again. Despite having a sclerotised cuticle and closing mechanisms on its spiracles, the beetle loses water by transpiration nearly as rapidly as do the other stages but, even so, keeps a fairly constant water content and water potential. Water stress has little effect on how long beetles survive or how many eggs they lay. They avoid burrowing into very dry or bare soil, and the females can delay laying their eggs if they are under water stress. When the female beetle does choose a site to lay its eggs, it burrows down into the soil without responding to vertical water potential gradients, just as the prepupal larva did before building the pupal cell. In this way both inactive stages (egg and pupa) are placed well below the surface of the soil and so avoid the extreme conditions which may develop near the surface.

The eggs are laid in clusters packed into the soil without any appreciable free space about them, and are bound together and to the soil by a secretion from the female beetle. The eggs rapidly absorb water during development by a process linked with their metabolism, which can take up water from soil against considerable water potential gradients. The eggs do not require contact with liquid water and hatch even in very dry soil.

The whole description of C. zealandica, its structure and its responses to water stress, portrays an animal which is very well adapted to the specialized soil environment. Its rank as a pest shows how well it survives the water stress and other hazards of this environment.

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